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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 :
C12N 15/13, 15/62, C07K 16/34, A61K 39/395, G01N 33/80

A1

(11) International Publicati n Number: **WO 97/49809**

(43) International Publication Date: 31 December 1997 (31.12.97)

(21) International Application Number: PCT/EP97/03253

(22) International Filing Date: 20 June 1997 (20.06.97)

(30) Priority Data:
96810421.6 24 June 1996 (24.06.96) EP
(34) Countries for which the regional or international application was filed: CH et al.

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: POLYPEPTIDES CAPABLE OF FORMING ANTIGEN BINDING STRUCTURES WITH SPECIFICITY FOR THE RHESUS D ANTIGENS, THE DNA ENCODING THEM AND THE PROCESS FOR THEIR PREPARATION AND USE

(57) Abstract

Polypeptides capable of forming antigen binding structures specific for Rhesus D antigens include the sequences indicated in the figures 1a to 16b. The obtained polypeptides, being Fab fragments, may be used directly as an active ingredient in pharmaceutical and diagnostic compositions. The Fab and their DNA sequences can also be used for the preparation of complete recombinant Anti-Rhesus D antibodies. Useful in pharmaceutical and diagnostic compositions.

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Polypeptides capable of forming antigen binding structures with specificity for the Rhesus D antigens, the DNA encoding them and the process for their preparation and use

This invention relates to polypeptides forming antigen binding structures with specificity for Rhesus D antigens and especially to Fab molecules with specificity for the Rhesus D antigen. The invention also relates to their application to provide pharmacological and diagnostic compositions. The above Fab fragments when genetically engineered to be part of complete antibodies are useful for the prophylaxis of hemolytic disease of the newborn (HDN). This invention provides the novel DNA and amino acid sequences of the above polypeptides.

Thus, the antibodies can be used for the protection of Rhesus negative women before or immediately after the birth of a Rhesus positive child to prevent HDN in subsequent pregnancies.

The invention also includes the application of the said Fab molecules either alone or in combination with Fc constant regions as complete antibodies for the purposes of treating other illnesses which might benefit from anti-Rhesus D immunoglobulin e.g. treatment of idiopathic thrombocytopenic purpura (ITP).

In addition anti-Rhesus D immunoglobulin can be used after mistransfusions of Rhesus positive blood to Rhesus negative recipients in order to prevent sensitization to the Rhesus D antigen. Further the invention relates to the application of these Fab fragments and antibodies as diagnostic reagents.

HDN is the general designation for hemolytic anemia of fetuses and newborn babies caused by antibodies of the mother. These antibodies are directed against antigens on the surface of the fetal erythrocytes. These antigens can belong to the Rhesus, ABO or other blood group systems.

The Rhesus blood group system includes 5 major antigens: D, C, c, E and e (Issitt, P.D., Med. Lab. Sci. 45:395, 1988). The D antigen is the most important of these antigens as it is highly immunogenic eliciting anti-Rhesus D antibodies during Rhesus incompatible pregnancies and following transfusion of Rhesus incompatible blood. The D antigen is found in approximately 85% of Caucasians in Europe and those individuals are said to

be Rhesus positive. Individuals lacking the D antigen are called Rhesus negative. The expression of the D antigen can vary due to either low antigen density, hereafter known as weak D or D^u, or due to partial antigenicity, hereafter known as partial D antigens.

The Rhesus D antigen, a membrane protein of the erythrocyte, has recently been cloned and its primary structure described (Le Van Kim, C., et al., PNAS 89:10925, 1992). Modeling studies suggest that the Rhesus D antigen has 12 transmembrane domains with only very short connecting regions extending outside the cell membrane or protruding into the cytoplasm.

The partial D phenotypes were first identified in people who carried D antigen on their red cells but who had an alloanti-D in their sera (Rose, R. R. and Sanger, R., Blood groups in man, Blackwell Scientific, Oxford, U.K. 1975; Tippett, P. et al., Vox Sanguinis. 70:123, 1996). This can be explained by regarding the D antigen as a mosaic structure with at least 9 different epitopes (epD1 to epD9). Thus in some D variant people the red cells lack part of this mosaic and antibodies are made to the missing D epitopes. Rhesus positive individuals that make antibodies against partial D antigens have been classified into six main different categories (D^{II} to D^{VII}) each having a different abnormality in the D antigen. More recently it has been shown that these D categories gave different patterns of reaction when tested against panels of human monoclonal anti-D antibodies (Tippett, P., et al., Vox Sanguinis. 70:123, 1996). The different reaction patterns identified the 9 epitopes and so define the different partial D categories. The number of epitopes present on the D antigen varies from one partial D category to another with the D^{VI} category expressing the least, epD3, 4 and 9. The D^{VI} category is clinically important as a D^{VI} woman can be immunized strongly enough to cause hemolytic disease of the newborn.

The prophylactic efficacy of anti-RhD IgG for prevention of hemolytic disease of the newborn is well established and has been in routine use for many years. As a result this severe disease has become a rarity. Nevertheless the underlying cause of the disease, i.e. RhD incompatibility between a RhD negative mother carrying a RhD positive child still remains and thus requires a continual supply of therapeutic anti-RhD IgG.

In recent years the assurance of a continual supply of anti-RhD IgG has become an increasing problem. The pool of available hyperimmune

serum from alloimmunized multiparous Rhesus negative women has drastically decreased due to the success of prophylactic anti-RhD. Thus the current methods of production require repeated immunization of an increasingly reluctant pool of donors for the production of high titer antiserum (Selinger, M., Br. J. Obstet. Gynaecol. 98:509, 1991). There are also associated risk factors and technical problems such as the use of Rhesus positive red blood cells for repeated immunization carrying the risk of transmission of viral diseases like hepatitis B, AIDS and other as yet unknown viruses (Hughes-Jones, N.C., Br. J. Haematol. 70:263, 1988). Therefore an alternative method for production of anti-RhD antibodies is required.

In the past few years various alternative sources of hyperimmune serum have been tried but all are associated with disadvantages. Epstein Barr Virus (EBV) transformation of lymphocytes creating B lymphoblastoid cell lines that secrete specific antibody including against the Rhesus D antigen (Crawford et al., Lancet. 386:Feb.19th, 1983) are unstable and require extensive cloning. Also due to the low transformation efficiencies (1-3% of B cells) only a restricted range of antibody specificities can be obtained from the potential repertoire. Additionally it seems that mice do not respond to the Rhesus D antigen and thus no murine monoclonal antibodies are available which could be used for producing chimaeric or humanised antibodies. Until recently the only other alternative was production of human antibodies by the hybridoma technique which was also restricted by the lack of a suitable human myeloma cell fusion partner (Kozbor, D. and Roder, J.C., Immunol. Today. 4:72, 1983).

It is thus the object of the present invention to provide Fab fragments having a reactivity against the Rhesus D antigen as well as complete antibodies comprising the Fab fragments which are free from the above mentioned drawbacks.

In the last few years the technique of repertoire cloning and the construction of phage display libraries has opened up new possibilities to produce human antibodies of defined specificity (Williamson, R.A. et al., PNAS 90:4141, 1993). These methods were thus applied to the preparation of polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens, especially of Fab fragments having an activity against Rhesus D and partial D antigens.

The generation of human antibodies by repertoire cloning as described in recent years (Barbas III, C.F. and Lerner, R.A., Companion Methods Enzymol. 2:119, 1991) is based on isolating mRNA from peripheral B cells. This method offers the tools to isolate natural antibodies, autoantibodies or antibodies generated during the course of an immune response (Zebedee, S.L., et al., PNAS 89:3175, 1992; Vogel, M. et al., Eur.J. Immunol. 24:1200, 1994). This method relies on constructing a recombinant antibody library from a particular donor starting from the mRNA coding for immunoglobulin (Ig) molecules. As only the peripheral blood lymphocytes (PBL) can be isolated from a donor the chances of finding specific antibody producing B cells in the periphery are increased if an individual is boosted with the desired antigen shortly before harvesting the PBL (Persson, M.A.A., et al., PNAS 88:2432, 1991). The total RNA is then isolated and the mRNA of the Ig repertoire can be cloned using Ig specific primers in the polymerase chain reaction (PCR) followed by the co-expression of heavy and light chains of the Ig molecule on the surface of a filamentous phage particle thereby forming an "organism" that in analogy to a B cell can bind to an antigen. In the literature this method is also known as the combinatorial approach as it allows the independent combining of heavy and light chains to form a functional Fab antibody fragment attached to one of the tail proteins, called pIII, of a filamentous phage. Phages carrying the Fab molecules (hereafter known as Phab particles) are selected for the desired antigen specificity, by a process known as bio-panning. The antigen can be applied to a solid support, specific Phab bind to the antigen whilst non specific Phab are washed away and finally the specific Phab are eluted from the solid support. The specific Phab are then amplified in bacteria, allowed to re-bind to the antigen on the solid support and the whole process of bio-panning is repeated.

The successive rounds of panning and amplification of selected Phab in bacteria result in an enrichment of specific Phab that can be seen from a rise in titer of colony forming units (cfu) plated out after each round of panning. Our previous experience and published data indicate that specific phage can usually be detected after 4 to 6 panning rounds (Vogel, M. et al., Eur.J. Immunol. 24:1200, 1994). In the above cited related art there is , however, no hint that the indicated steps can be used for a successful preparation of Fab fragments of anti-Rh D antibodies.

In the appended figures 1a to 16b; DNA sequences coding for variable regions (V regions) of anti Rh D Fab fragments and the corresponding polypeptide sequences are disclosed.

Fig. 17 shows the pComb3 expression system used according to the present invention.

Figs. 18 and 19 show the separate preparation of genes of the heavy and light chains of the complete antibody according to the description in example 6.

Subjects of the present invention are polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens according to the definition of claim 1. The table in claim 1 refers to the appended figures. The identification number for each sequence is given. The locations of the Rhesus D specific CDR1 (complementarity determining region 1), CDR2 and CDR3 regions are indicated in the figures and according to base pair number in the table of claim 1. Preferred polypeptides according to the invention are anti-Rhesus D antibodies which include the variable regions of the heavy and light chains according to the sequences given in Figs. 1a -16b. The Figs. 1a, 2a, ... 16a are related to the variable regions of the heavy chain and the Figs. 1b, 2b, ... 16b are related to the variable regions of the light chain.

Further subjects of the present invention are the DNA sequences coding for antigen binding polypeptides according to the definition of claim 6. Prefered DNA sequences are those coding for variable regions of Fab fragments of anti-Rh D antibodies according to the Figs. 1a -16b. The Figs. 1a, 2a, ... 16a are related to the heavy chain and the Figs. 1b, 2b, ... 16b are related to the light chain.

A further subject of the present invention is a process for preparing recombinant Fab polypeptides according to the definition in claim 11.

A further subject of the present invention is a process for the selection of recombinant polypeptides according to claim 12.

Further subjects of the present invention are anti-Rh D antibodies according to the definition of claim 14, preferably anti-Rh D immunoglobulin molecules comprising the heavy and light chain variable regions according to

the Figs. 1a to 16b combined with known heavy and light chain constant regions.

Further subjects of the present invention are pharmaceutical and diagnostic compositions comprising polypeptides, anti-Rh D antibodies or Fab fragments according to the invention.

The total re-amplified Phab population obtained after each panning can be tested for specificity using various methods such as ELISA and immunodot assays. It is also defined by the nature of the antigen e.g. anti-Rhesus D Phabs are detected by indirect haemagglutination using a rabbit anti-phage antibody or equivalent Coombs reagent as the cross linking antibody. Once a total Phab population has been identified as positive for the desired antigen, individual Phab clones are isolated and the DNA coding for the desired Fab molecules is sequenced. Individual Fab can then be produced by use of the pComb3 expression system which is illustrated in Fig. 16. In this system the gIII gene, coding for the tail protein pIII, is cut out from the phagemid vector pComb3. This allows production of soluble Fab in the bacterial periplasm. Such individual Fab fragments can then be tested for antigen specificity.

The phage display approach has also been used as a means of rescuing monoclonal antibodies from unstable hybridoma cell lines. This has been reported for anti-Rhesus D antibodies (Siegel, D.L. and Silberstein, L.E., Blood. 83:2334, 1994; Dziegiel, M. et al., J. Immunol. Methods. 182:7, 1995). A phage display library constructed from non-immunized donors has also been used to select Fv fragments (i.e. variable regions of heavy and light chains, V_H and V_L) specific for human blood group antigens which included one Fv fragment reacting against the Rhesus D antigen (Marks, J.D. et al., Biotechnology. 11:1145, 1993).

Important considerations when constructing combinatorial libraries are the source of cells used for RNA extraction and the nature of the antigen used for panning. Therefore, this invention uses a hyperimmune donor who was boosted i.v. with Rhesus D^+ red blood cells (rbc). The PBL of the donor were harvested at +5 and +18 days after the i.v. boost and were used to construct 2 combinatorial libraries hereafter known as library D1 (LD1) and library D2 (LD2) respectively. Double immunofluorescence analysis of the harvested PBL, using the markers CD20 and CD38 for pan B cells and

lymphoblastoid cells respectively, showed a higher than normal percentage of lymphoblastoid B cells, of plasma cell morphology. The high number of plasma cells found in the peripheral blood is most unusual as normally there are less than 1% in the periphery and probably indicates that the donor had a high percentage of circulating B cells with specificity for the Rhesus D antigen.

After construction of the library, the selection of Phabs specific for the Rhesus D antigen was achieved by bio-panning on fresh whole rbc of phenotype R1R1 (CDe/CDe) i.e. the reference cells used for Rhesus D typing. This was necessary since the Rhesus D antigen, an integral membrane protein of 417 amino acids (Le Van Kim, C. et al, PNAS 89:10925, 1992), loses its immunogenicity during purification (Paradis, G. et al, J. Immunol. 137:240. 1986) and therefore a chemically purified D antigen cannot be bound to a solid phase for selection of immunoreactive Phabs as for other antigen specificities previously selected in this system (Vogel, M. et al., Eur.J. Immunol. 24:1200, 1994). Modelling studies have suggested that only very short connecting regions of the Rhesus D antigen extend outside the cell membrane or protrude into the cytoplasm (Chérif-Zahar, B. et al, PNAS 87:6243, 1990). Thus the parts of the RhD antigen visible to antibodies are relatively restricted and may be under conformational constraint. This aspect of the Rhesus D antigen becomes even more important when considering selection of Phabs with reactivity against the partial D phenotypes which essentially lack certain defined epitopes of the D membrane protein (Mouro, I. et al, Blood. 83:1129, 1994).

Furthermore, since whole rbc do not only express the D antigen, a series of negative absorptions had to be performed on Rhesus D negative rbc in order to absorb out those Phabs reacting with the other antigenic proteins found on the rbc.

This panning procedure performed on Phabs coming from both LD1 and LD2 librairies resulted in the isolation of 6 different Fab producing clones from library LD1, 8 different Fab producing clones from library LD2 and 2 Fab producing clones from the pooled libraries LD1 and LD2.

The nomenclature and the figures where the sequences are listed are given in table 1.

Table 1

LIBRARY LD1 Clone No.	V_H- Sequence Figure	V_L- Sequence Figure	LIBRARY LD2 Clone No.	V_H- Sequence Figure	V_L- Sequence Figure
LD1-40	1a	1b	LD2-1	6a	6b
LD1-52	2a	2b	LD2-4	7a	7b
LD1-84	3a	3b	LD2-5	8a	8b
LD1-110	4a	4b	LD2-10	9a	9b
LD1-117	5a	5b	LD2-11	10a	10b
			LD2-14	11a	11b
			LD2-17	12a	12b
			LD2-20	13a	13b

The above Fab clones show exclusive reactivity against the Rhesus D antigen, 3 of 5 D^u rbc tested and agglutinating reactivity against the Partial D phenotypes as follows: Rh33, DIII, DIVa, DIVb, DVa, DVII,.

However, using the above mentioned R1R1 rbc for panning of the Phabs, no clones were isolated which reacted against the Partial DVI phenotype. As the serum of the original hyperimmune donor tested at the time of construction of the recombinant library, was known to react against the DVI phenotype the recombinant library should also contain the anti-DVI specificity.

In order to select for the DVI reactivity the panning conditions were changed in that different cells were used. A special donor whose rbc had been typed and were known to express the Partial DVI phenotype was used as the source of cells for re-panning the LD1 and LD2 libraries. This second series of pannings was essentially performed in the same way as the first series except for the substitution of DVI rbc for R1R1 rbc and the addition of bromelase treatment to the DVI rbc. The DVI phenotype expresses the least number of Rhesus D epitopes and it is therefore difficult to make antibodies against it. It has been reported that only 15% of unselected polyclonal anti-D and 35% of selected anti-D made by Rhesus D negative subjects reacted with DVI+ cells (Mouro, I. et al, Blood. 83:1129, 1994). Bromelase treatment which removes N- acetylneuraminic acid (sialic acid) from the rbc membrane, was performed in order to render the Rhesus DVI epitopes more accessible during the panning with the pre-absorbed Phabs.

This second series of pannings on the LD1 library resulted in 1 Fab producing clone LD1-6-17. The nomenclature is given in table 2.

Table 2

LIBRARY LD1	V_H -Sequence figure	V_L-Sequence figure
Clone No: LD1-6-17	14a	14b

However this clone was reacting with Rhesus alleles C and E and showing a false positive reaction with DVI positive rbc. This was also due to the phenotype of the DVI donor (Cc DVI ee) who expressed the C allele which was not absorbed out by the Rhesus negative rbc (ccddee).

Thus a third series of pannings on a pool of the LD1 and LD2 libraries was performed using different rbc for the absorption phase. After 6 rounds of panning using both bromelase treated and non treated rbc for both the absorption steps and the elution from DVI positive rbc a total population of Phabs was obtained which reacted exclusively with rbc of phenotype R1R1 (CCDDee) and 2 different donors expressing the DVI variant.

This third series of pannings on the LD1 and LD2 librairies resulted in 2 Fab producing clones reacting with DVI+ rbc. The nomenclature is given in table 3.

Table 3

LIBRARY LD1/LD2	V_H -Sequence figure	V_L-Sequence figure
Clone No: LD1/2-6-3	15a	15b
Clone No: LD1/2-6-33	16a	16b

Thus a total of 16 different anti-Rhesus D Fab clones have been isolated. The DNA from these clones has been isolated and sequenced using Fluorescent Cycle Sequencing on an ABI 373A Sequencing System. The nucleotide and corresponding amino acid sequences of the said Fab clones form the basis of this invention.

Sequence analysis has revealed that several clones were isolated bearing the same V_H gene segment but different V_L gene segments. This is

the case for the two clones LD2-1 and LD2-10, for the two clones LD2-4 and LD2-11, and for the three clones LD2-14, LD1/2-6-3 and LD1/2-6-33, respectively.

The DNA sequences obtained and Fab fragments are useful for the preparation of complete antibodies having an activity against the Rhesus D antigen. Suitable expression systems for such antibodies are mouse myeloma cells or chinese hamster ovary cells.

The examples which follow explain the invention in detail, without any restriction of the scope of the invention.

Example 1 describes the construction of 2 combinatorial librairies; especially the aforementioned LD1 and LD2 libraries.

Example 2 describes a series of pannings using R1R1 rbc on the said LD1 and LD2 libraries in detail.

Example 3 describes a series of pannings using both bromelase and non bromelase treated rbc for absorption and bromelase treated DVI positive rbc using a pool of the said LD1 and LD2 librairies.

Example 4 describes an indirect haemagglutination assay using a rabbit anti-phage antibody, as an equivalent Coombs reagent, to monitor the enrichment and specificity of Rhesus D specific Phabs after panning.

Example 5 describes the preparation and purification of Fab antibody fragments for application as diagnostic reagents.

Example 6 describes the preparation of complete anti-Rhesus D immunoglobulins using the sequences of the present invention.

Example 1

Construction of the recombinant LD1 and LD2 libraries

a) Source of the lymphocytes

A male adult who was a member of the volunteer pool of hyperimmune Rhesus D donors was given an i.v. boost of 2 ml of packed rbc from a known male donor of blood group O RhD$^+$. The PBL were harvested at +5 and +18 days after the boost and the mononuclear cells (MNC) isolated by Ficoll gradient centrifugation (Lymphoprep, Pharmacia, Milwaukee, WI). The results of donor lymphocyte analysis of day +5 are given in table 4. The +5 day MNC were used directly for RNA preparation using a phenol-chloroform guanidinium isothiocyanate procedure (Chomczynski, P. and Sacchi, N., Anal. Biochem. 162:156, 1987). The +18 day MNC were first cultured for 3 days in RPMI-1640 medium (Seromed, Basel) containing 10^3 U/ml of IL-2 (Sandoz Research Center, Vienna, Austria) and 10 µg/ml of pokeweed mitogen (PWM; Sigma L9379, Buchs, Switzerland) before extracting RNA.

Table 4

Immunofluorescence analysis of donor lymphocytes +5 days after rbc i.v. boost

Cell surface antigen	% Positive cells	Cell surface antigen	% Positive cells
CD20	15	CD8	12
CD38	20	CD25	7.6
CD20/38	15	CD57	12.5
CD3	47	CD14	6
CD4	34	HLA-DR	18

b) Construction of Library

Two separate libraries were constructed called LD1 and LD2 (as detailed above) corresponding to the cells harvested at +5 days and +18 days (finally +21 days including the +3 days PWM stimulation) after the i.v. boost respectively. Total RNA was then prepared from these cells using a phenol-chloroform guanidinium isothiocyanate method. From this RNA, 10 µg were

used to make cDNA using an oligo(dT) primer (400 ng) and reverse transcribed with M-MuLV reverse transcriptase according to the conditions specified by the supplier (Boehringer Mannheim Germany). PCR amplification was performed as described in Vogel, M. et al., E.J. of Immunol. 24:1200, 1994. Briefly, 100 µl PCR reaction contained Perkin-Elmer buffer with 10 mM $MgCl_2$, 5 µl cDNA, 150 ng of each appropriate 5′ and 3′ primer, all four dNTP at 200 µM each and 2 U/ml Taq Polymerase (Perkin Elmer, NJ). The PCR amplification of the heavy and light chains of the Fab molecule was performed separately with a set of primers from Stratacyte (details given below). For the heavy chain six upstream primers were used that hybridize to each of the six families of the V_H genes whereas one kappa and one lambda chain primer were used for the light chain. The downstream primers were designed to match the hinge region of the constant domains γ1 and γ3 for the heavy chain. For the light chain the downstream primers were matched to the 3′ end of kappa and lambda constant domains. The heavy and light chain PCR products were pooled separately, gel purified and cut with Xho1/Spe1 and Sac1/ Xba1 restriction enzymes (Boehringer Mannheim), respectively. After digestion the PCR products were extracted once with phenol : chloroform : isoamylalcohol and purified by gel excision. The insertion of the Xho1/Spe1 digested Fd fragment and subsequent ligation of the Sac1/Xba1 digested light chain into the pComb3 vector, the transformation into XL1-Blue cells, and the production of phages were performed as described by (Barbas III, C.F. and Lerner, R.A., Companion Methods Enzymol. 2:119, 1991).

After transformation of the XL1-Blue E.coli cells samples were withdrawn and titrated on plates to determine the library size. These results indicated expression libraries of $7.5x10^6$ and $7.7x10^6$ cfu (colony forming units) for LD1 and LD2 respectively.

c) PCR Primers

VHI 5'-CAC TCC CAG GTG CAG CTG CTC GAG TCT GG-3'
VHII 5'-GTG CTG TCC CAG GTC AAC TTA CTC GAG TCT GG-3'
VHIII 5'-GTC CAG GTG GAG GTG CAG CTG CTC GAG TCT GG-3'
VHIV 5'-GTC CTG TCC CAG GTG CAG CTG CTC GAG TCG GG-3'
VHV 5'-GTC TGT GCC GAG GTG CAG CTG CTC GAG TCT GG-3'
VHVI 5'-GTC CTG TCA CAG GTA CAG CTG CTC GAG TCA GG-3'
CHI(gI) 5'-AGC ATC ACT AGT ACA AGA TTT GGG CTC-3'

VL(k) 5'-GT GCG AGA TGT GAG CTC GTG ATG ACC CAG TCT CCA-3'
CL(k) 5'-T CCT TCT AGA TTA CTA ACA CTC TCC CCT GTT GAA GCT CTT TGT GAC GGG CGA ACT C-3'
VL(l) 5'C TGC ACA GGG TCC TGG GCC GAG CTC GTG GTG ACT CA-3'
CL(l) 5'G CAT TCT AGA CTA TTA TGA ACA TTC TGT AGG GGC-3'

d) Vectors and bacterial strains

The pComb3 vector used for cloning of the Fd and the light chain was obtained from the Scripps Research Institute La Jolla, CA; (Barbas III, C.F. and Lerner, R.A., Companion Methods Enzymol. 2:119, 1991). The *Escherichia coli* strain XL1-Blue used for transformation of the pComb3 vector and the VCSM13 helper phage were purchased from Stratacyte (La Jolla, CA).

Example 2

Selection of Rhesus D Phabs from LD1 and LD2 libraries on R1R1 rbc

a) Absorption and Bio-Panning

A series of three negative absorptions on rbc group O Rh negative were performed for each panning round before positive selection on rbc group O Rh positive (R1R1). Fresh rbc were collected in ACD (acid citrate dextrose) anticoagulant and washed 3 times in 0.9% NaCl. The rbc were counted in Hayems solution and adjusted to $40x10^6$/ml. Absorption : 1 ml of phage preparation in PBS/3%BSA was added to rbc group O Rh negative pellet ($16x10^6$ rbc) in 12 ml tubes (Greiner 187261, Reinach, Switzerland) and incubated at RT for 30 min. with careful shaking. All tubes were pre-blocked in PBS/3% BSA for a minimum of 1hr at RT. The rbc were pelleted by centrifuging for 5 min. 300 x g at 4°C. The resulting phage supernatant was carefully harvested and the process repeated twice more. After the final absorption the phage supernatant was added to the rbc group O Rh positive pellet ($16x10^6$ rbc) and again incubated at RT for 30 min. with gentle shaking. Then the rbc were washed at least 5 times in 10 ml ice cold PBS, centrifuged 5 min. 300 x g at 4°C, followed by elution with 200 µl of 76 mM citric acid pH 2.8 for 6 min. at R.T. and neutralisation with 200 µl 1M Tris. The rbc were centrifuged 300 x g, 5 min. at 4°C and the resulting supernatant containing the eluted phages was carefully removed and stored with carrier protein

(0.3% BSA) at 4°C ready for re-amplification. The numbers of Rhesus D specific Phabs of each panning round are given in table 5.

Table 5

Selection of Rhesus D+ Phabs from the LD1 and LD2 libraries on R1R1 rbc

	No. of eluted Rhesus D specific phages	
Panning Round No.[a)]	**Library D1 cfu**	**Library D2 cfu**
1	$8x10^6$	$4.6x10^7$
2	$6x10^7$	$1.4x10^7$
3	$1x10^8$	$7.9x10^7$
4	$3x10^8$	$1.3x10^8$
5	$3x10^8$	$1x10^8$
6	nd	$2.8x10^8$

a) For each round 10^{12} Phabs were incubated in tubes with rbc Group O Rhesus negative (absorption phase) followed by elution from rbc Group O Rhesus positive (R1R1)

nd = not done
cfu = colony forming units

Example 3

Selection of Rhesus D Phabs from the pooled LD1 and LD2 libraries on DVI+ rbc

a) Absorption on rbc group O Rh negative, phenotypes 1 (r'r, Ccddee) and 2 (ryry, CCddEE)

A series of four negative absorptions on rbc group O Rh negative was performed for each panning round before positive selection on rbc group O Rh DVI positive. The negative absorptions were performed in the following order : Step 1) phenotype 1 treated with bromelase; step 2) phenotype 1 no bromelase; step 3) phenotype 2 treated with bromelase; step 4) phenotype 2

no bromelase. Frozen rbc were thawed into a mixture of sorbit and phosphate buffered saline, left standing in this solution for a minimum of 10 min. and then washed 5 to 6 times in phosphate buffered saline and finally stored in stabilising solution (DiaMed EC-Solution) ready for use. Before panning the rbc were washed 3 times in 0.9% NaCl. followed by counting in Hayems solution. Absorption : 1 ml of phage preparation in PBS/3%BSA was added to an rbc pellet ($2x10^8$) as in step 1 in 12 ml tubes (Greiner 187261, Reinach, Switzerland) and incubated at RT for 30 min. with careful shaking. All tubes were pre-blocked in PBS/3% BSA for a minimum of 1hr at RT. The rbc were pelleted by centrifuging for 5 min. 300 x g at 4°C. The resulting phage supernatant was carefully harvested and the process repeated using rbc as detailed above in steps 2, 3, and 4.

b) Treatment of rbc Rhesus D negative r'r and ryry and Rhesus DVI+ with bromelase

Bromelase 30 (Baxter, Düdingen, Switzerland) was used to treat rbc Rhesus DVI+ in the same proportions as used in a routine haemagglutination assay, i.e. 10 µl bromelase per $2x10^6$ rbc. Thus bromelase was added to the required amount of rbc and incubated at 37°C for 30 min. followed by washing 3 times in 0.9% NaCl, re-counting in Hayems solution and adjusting to the required concentration in PBS/3% BSA ready for Phab panning.

c) Bio-Panning on bromelase treated Rhesus DVI+ rbc

After the final absorption on rbc ryry non bromelase treated the phage supernatant was divided into 2 equal parts and added either to the enzyme or non enzyme treated rbc group O Rh DVI+ pellet ($40x10^6$) respectively and again incubated at RT for 30 min. with gentle shaking. Then the 2 populations of rbc were washed at least 5 times in 10 ml ice cold PBS, centrifuged 5 min. 300 x g at 4°C, followed by elution with 200 µl of 76 mM citric acid pH 2.8 for 6 min. at R.T. and neutralisation with 200 µl 1M Tris. The rbc were centrifuged 300 x g, 5 min. at 4°C and the resulting supernatants containing the eluted phages from either the bromelase or non bromelase treated DVI+rbc were carefully removed and stored with carrier protein (0.3% BSA) at 4°C ready for re-amplification. In further rounds of panning the eluted phage from either the bromelase or non bromelase treated DVI+ rbc were

kept separate and each followed the absorption protocol steps 1 to 4. The elution step was slightly different compared to panning round 1 as the phage populations were not again divided into 2 parts. Only those phage eluted from bromelase treated DVI+ rbc were also eluted again from bromelase treated DVI+ rbc and only those phage eluted from the non bromelase treated DVI+ rbc were also again eluted from non bromelase treated DVI+ rbc. The numbers of specific Phabs after each panning round are given in table 6.

Table 6 **Selection of Rhesus D Phabs from pooled LD1 and LD2 libraries on Rhesus DVI+ red blood cells**

	No. of eluted Rhesus DVI+ specific phages	
Panning Round No.a)	- Bromelase cfu	+ Bromelase cfu
1	$1.9x10^6$	$4.4x10^6$
2	$1.6x10^6$	$4x10^5$
3	$2.4x10^7$	$4.1x10^7$
4	$3x10^6$	$5x10^7$
5	$1x107^8$	$1x10^8$
6	nd	$3x10^8$

a) For each round 10^{12} Phabs were incubated in tubes with 2 different phenotypes of rbc Group O Rhesus negative (absorption phase) followed by elution from rbc Group O Rhesus DVI+.

Example 4

Monitoring of the panning rounds and determination of the specificity of the enriched Phabs using a rabbit anti-phage antibody

Indirect haemagglutination assay

Freshly collected rbc of different ABO and Rhesus blood groups were washed 3 times in 0.9% NaCl and adjusted to a 3-5% solution (45-50x10^7/ml) in either 0.9% NaCl or PBS/3% BSA. For each test condition 50 µl rbc and 100 µl test (precipitated and amplified phage or control antibodies) were incubated together in glass blood grouping tubes (Baxter, Düdingen, Switzerland) for 30 min. at 37°C. The rbc were washed 3 times in 0.9% NaCl

and then incubated with 2 drops of Coombs reagent (Baxter, Düdingen, Switzerland) for positive controls or with 100 µl of 1/1000 diluted rabbit anti-phage antibodies (made by immunising rabbits with phage VCSM13 preparation, followed by purification on an Affi-Gel Blue column and absorption on E. coli to remove E. coli-specific antibodies). The tubes were incubated for 20 min at 37°C, centrifuged 1 min at 125xg and rbc examined for agglutination by careful shaking and using a magnifier viewer.

When purified Fab were tested for agglutination, an affinity purified anti-Fab antibody (The Binding Site, Birmingham, U.K.) was used instead of the rabbit anti-phage antibody.

Table 7 shows the results of haemagglutination tests of Phab samples after different panning rounds on R1R1 rbc.

Table 8 shows the results of haemagglutination tests of Phab samples after different panning rounds on Rhesus DVI+ rbc.

Table 9 shows the reactivity pattern of individual Fab clones from libraries LD1 and LD2 with partial D variants.

Table 7 **Monitoring of Phabs from LD1 and LD2 libraries by indirect haemagglutination after panning on R1R1 rbc**

Phab sample Panning round	Library LD1 tested on rbc O Rh D+ (a)	Library LD2
No. 4		
undiluted	+	+
1/4	+	+/-
1/20	-	-
No.5		
undiluted	++	+
1/4	++	+
1/20	-	-
No. 6		
undiluted	nd	+++
1/4	nd	++
1/20	nd	nd
Helper phage (b) undiluted, 1/4, 1/20	-	-

a) Indirect haemagglutination was performed in glass tubes using 50 µl rbc ($40x10^7$/ml) and 100 µl Phabs starting at $4x10^{11}$/ml. After 30 min. at 37°C the

rbc were washed 3 times and further incubated for 20 min. at 37°C with a 1/1000 dilution of rabbit anti-phage antibody.

b) The M13 helper phage was used as a negative control and showed no non-specific agglutination due to the phage particle alone.

Agglutination was scored by visual assessment from +++ (strong agglutination) descending to - (no agglutination). nd = not done

Table 8 **Monitoring of Phabs from pooled LD1 and LD2 libraries by indirect haemagglutination after panning on Rhesus DVI+ rbc**

Phab sample Panning round	rbc phenotypes					
	CCDDee	ccddee	Ccddee	CCddEE	DVI (E.J.)	DVI (K.S.)
Non Bromelase treated rbc DVI+						
Round No.3	[a] +++	-	+/-	(+)	+/-	+/-
Round No. 5	++	-	-	-	-	-
Bromelase treated rbc DVI+						
Round No.4	+++	-	+/-	-	(+)	+/-
Round No.5	+++	-	+/-	+/-	(+++)	++
Round No.6	++++	-	-	-	+++	+++
LD1 - 6 - 17	reactive with C and E					
LD1/2 - 6 - 3	++++	-	-	-	+/-	nd
LD1/2 - 6 - 33	++++	-	-	-	+	nd

a) Agglutination was scored by visual assessment from ++++ (strong agglutination) descending to - (no agglutination). nd = not done

Note: Only those Phabs eluted from bromelase treated DVI+ rbc showed evidence of agglutination against 2 different DVI+ donors.

Table 9

Clonal Analysis of Reactivity of Fab anti-Rh sus D Clones from Libraries D1 and LD2 against Partial D Variants

		Partial D Variants					
[a] Fab Clone No	Rh33	DIII	DIVa	DIVb	DVa	DVI	DVII
LD1 - 40	-	[b]+++	+	+	+/-	-	++
- 52	-	+++	-	-	+++	-	+++
- 84	-	++	-	-	-	-	+
- 110	(+)	+++	++	+	+	-	++
- 117	-	+++	-	-	-	-	++
LD2 - 1	+++	nd	+++	+++	+	-	+++
- 4	-	+++	-	+	-	-	+
- 5	-	nd	+++	+++	-	-	+++
- 10	(-)	+++	+++	+++	+	-	++
- 11	-	+++	-	-	-	-	++
- 14	+++	+++	+++	+++	+++	-	+++
- 17	-	+++	+++	+	+/-	-	+++
- 20	-	+++	+++	-	+/-	-	+++
LD1/2 - 6- 3	++	+++	+++	++	+++	+	++
LD1/2 - 6- 33	+/-	+++	+++	++	+++	+	++

a) soluble Fab preparations were made of each clone followed by indirect haemagglutination.

b) Agglutination was scored by visual assessment from +++ (all cells agglutinated in a clump) descending to - (no cells agglutinated).

Example 5

Preparation and purification of Fab antibody fragments for application as diagnostic reagents

After the bio-panning procedures detailed in Examples 2 and 3 a phage population which showed specific agglutination on Rhesus D+ rbc was selected and used to prepare phagemid DNA. More precisely the Phabs selected on R1R1 rbc were used after the 5th and 6th rounds of bio-panning for LD1 and LD2 libraries respectively and after the 5th bio-panning on DVI+ rbc for isolation of the LD1-6-17 clone. In order to produce soluble Fab, the sequence gIII coding for the pIII tail protein of the phage particle must be deleted.

Phagemid DNA was prepared using a Nucleotrap kit (Machery-Nagel) and the gIII sequence was removed by digesting the so isolated phagemid DNA with Nhe1/Spe1 as described (Burton, D.R., et al.,PNAS, 1989). After transformation into XL1-Blue individual clones were selected (nomenclature given in table 1) and grown in LB (Luria Broth) containing 50 μg/ml carbenicillin at 37°C to an OD of 0.6 at 600 nm. Cultures were induced with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Biofinex, Praroman, Switzerland) and grown overnight at 37°C. The whole culture was spun at 10,000xg for 30 min. at 4°C to pellet the bacteria. The bacterial pellet was treated with a lysozyme/DNase solution to liberate the Fab fragments inside the cells. As some Fab were released into the culture supernatant this was also harvested separately. These Fab preparations were then pooled and precipitated with 60% ammonium sulphate (Merck, Darmstadt, Germany) to concentrate the Fab followed by extensive dialysis in phosphate buffered saline (PBS) and ultracentrifugation at 200,000xg to pellet any insoluble complexes. The Fab preparations were then purified on a ceramic hydroxyapatite column (HTP Econo cartridge, BioRad, Glattbrugg, Switzerland) using a gradient elution of PBS (Buffer A) and PBS + 0.5M NaCl (Buffer B). The linear gradient was programmed to increase from 0-100% Buffer B in 40 min. The Fab was eluted as a single peak between 40-60% Buffer B. The positive fractions as identified by immunodot assay using an anti-Fab peroxidase conjugate (The Binding Site, Birmingham, U.K.) were pooled, concentrated using polyethylene glycol and extensively dialysed

against PBS. The positive fractions from the hydroxyapatite column for each clone were used in a classical indirect haemagglutination assay in glass tubes using either the standard Coombs reagent (Baxter Diagnostics AG Dade, anti-human serum) or an anti-Fab (The Binding Site, Birmingham, U.K.) as the cross linking reagent. These Fab of defined specificity on the Partial D variants as shown on page 18 can be used to type rbc of unknown Partial D phenotype.

Example 6

Construction of complete immunoglobulin genes

The LD2-14 heavy chain V gene (V_H gene) was amplified from the anti-Rhesus D-Fab-encoding plasmid LD2-14 with the polymerase chain reaction (PCR) using specific primers. The 5'-primer had the sequence:

5'-GGGTCGACGCACAGGTGAAACTGCTCGAGTCTGG-3',

whereas the 3'-primer was of the sequence:

5'-GCCGATGTGTAAGGTGACCGTGGTCCCCTTG-3'.

The PCR reaction was performed with Deep Vent DNA Polymerase and the buffer solution (2mM Mg^{++}) from New England Biolabs at the conditions recommended by the manufacturer including 100 pmol of each primer and the four deoxynucleotides at a concentration of 250 µM each. The reaction was run for 30 cycles with the following temperature steps: 60 s at 94°C (extended by 2 min. during the first cycle), 60 s at 57°C and 60 s at 72°C (extended by 10 min. during the last cycle). Post-amplification addition of 3' A-overhangs was accomplished by a subsequent incubation for 10 min at 72°C in the presence of 1 unit Taq DNA Polymerase (Boehringer Mannheim, Germany). The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Switzerland) and cloned into the vector pCRII using Invitrogen's TA cloning kit (San Diego, USA). Having digested the resulting plasmid TAVH14 with *Sal*I and *Bst*EII, the V_H gene was isolated by preparative agarose gel electrophoresis using Qiagen's QIAquick gel extraction kit.

Vector # 150 (Sandoz Pharma, Basel) which contained an irrelevant but intact human genomic immunoglobulin V_H gene was cut with

*Sal*I and *Bst*EII, and the vector fragment was isolated by preparative agarose gel electrophoresis using Qiagen's QIAquick gel extraction kit. Ligation of vector and PCR product was performed at 25°C for 2 hours in a total volume of 20 µl using the rapid DNA Ligation kit (Boehringer Mannheim, Germany). Following ligation, the reaction mix was diluted with 20 µl H_2O and extracted with 10 volumes of n-butanol to remove salts. The DNA was then pelleted by centrifugation, vacuum dried and resuspended in 10 µl H_2O. 5 µl of this DNA solution were electroporated (0.1 cm cuvettes, 1.9 kV, 200 Ω, 25 µFD) with a GenePulser (BioRad, Gaithersburg) into 40 µl of electroporation competent E. coli XL1-blue MRF' (Stratagene, La Jolla), diluted with SOC medium, incubated at 37°C for 1 hour and plated on LB plates containing ampicillin (50 µg/ml). Plasmid-minipreps (Qiagen, Basel) of the resulting colonies were checked with restriction digests for the presence of the appropriate insert.

With this procedure, the irrelevant resident V_H gene in vector # 150 was replaced by the amplified anti-Rhesus D V_H sequence of LD2-14 and yielded plasmid cassVH14. The structure of the resulting immunoglobulin V_H gene construct was confirmed by sequencing, cut out by digestion with *Eco*RI and *Bam*HI and gel purified as described above. Expression vector # 10 (Sandoz Pharma, Basel) containing the human genomic immunoglobulin Cγ1 gene segment was also digested with *Eco*RI and *Bam*HI, isolated by preparative agarose gel electrophoresis, ligated with the *Eco*RI / *Bam*HI-V_H gene segment previously obtained from plasmid cassVH14 and electroporated into E. coli XL1-blue MRF' as outlined above. This resulted in a complete anti-Rhesus D heavy chain immunoglobulin gene in the expression vector 14IgG1 (Figure and).

The LD2-14 light chain V gene (V_L gene) was amplified from the same anti-Rhesus D-Fab plasmid LD2-14 by PCR using specific primers. The 5'-primer had the sequence:

5'-TACGCGTTGTGACATCGTGATGACCCAGTCTCCAT-3',

whereas the 3'-primer was of the sequence:

5'-AGTCGCTCAGTTCGTTTGATTTCAAGCTTGGTCC-3'.

PCR reaction, product purification and subsequent cloning steps were analogous to the steps described for the V_H gene, except that the appropriate light chain vectors were used. Briefly, the V_L PCR product was

cloned into pCRII vector yielding plasmid TAVL14, excised therefrom with *Mlu*I and *Hin*dIII and isolated by gel extraction. The V_L gene was subsequently cloned into the *Mlu*I and *Hin*dIII sites of vector # 151 (Sandoz Pharma, Basel) thus replacing the irrelevant resident V_L gene by the amplified anti-Rhesus D V_L sequence of LD2-14. Having confirmed the sequence of the resulting plasmid cassVL-14, the *Eco*RI / *Xba*I fragment containing the V_L gene was then subcloned into the restriction sites *Eco*RI and *Xba*I of vector # 98 (Sandoz Pharma, Basel, Switzerland) which contains the human genomic immunoglobulin Cκ gene segment. This procedure replaced the irrelevant resident V_L gene in plasmid # 98 and yielded the expression vector 14kappa which contains the complete anti-Rhesus D light chain immunoglobulin gene.

The mouse myeloma cell line SP2/0-Ag 14 (ATCC CRL 1581) was cotransfected by electroporation with the expression vectors 14IgG1 and 14kappa previously linearized at the unique *Eco*RI and *Not*I cleavage site, respectively. The electroporation was performed as follows: exponentially growing cells were washed twice and suspended in phosphate buffered sucrose (272 mM sucrose, 1 mM $MgCl_2$, 7 mM NaH_2PO_4, pH 7.4) at a density of 2×10^7 cells/ml. 0.8 ml of cells were added to a 0.4 cm cuvette, mixed with 15 µg of linearized plasmids 14IgG1 and 14kappa, held on ice for 15 min., electroporated with 290 Volts, 200 Ω, 25 µFD, put back on ice for 15 min., transferred to a T75 cell culture flask with 20 ml of cold RPMI 1640 medium (10% heat inactivated fetal bovine serum, 50 µM beta-mercaptoethanol), left for 2 h at room temperature and then incubated for 60 h at 37°C. After this period, the cells were transferred to 50 ml of medium containing 1 mg/ml G418 for selection. Stable transfectants were then selected in the presence of increasing concentrations of methotrexate to amplify the integrated DNA and thus increasing the expression of the corresponding antibody rD2-14.

Expression of rD2-14 in the culture's supernatant (SrD2-14) was monitored by an enzyme linked immuno-sorbent assay (ELISA) specific for human γ1 and kappa chains. Quantification of the Rhesus D specific immunoglobulins in the anti-D assay according to Ph. Eur. revealed between 1.1 and 11.4 µg/ml of agglutinating antibody in such supernatants. They tested agglutination negative for Rhesus negative rbc and revealed the same agglutination potential against partial D variants as the Fab LD2-14 expressed in E. coli. The data are shown in table 10.

Table 10

Comparativ analysis of reactivity of Fab anti-Rhesus D clone LD2-14 and antibody rD2-14 against partial D variants

			Partial D Variants						
	R1R1	rr	Rh33	DIII	DIVa	DIVb	DVa	DVI	DVII
LD2-14	+++	-	+++	+++	+++	+++	+++	-	+++
SrD2-14	+++	-	+++	+++	+++	+++	+++	-	+++
TCB	-	-							

Agglutination was scored by visual assessement from +++ (all cells agglutinated in a clump) descending to - (no cells agglutinated).

LD2-14: Fab fragment prepared as described in Example 5;
SrD2-14: cell culture supernatant containing antibody rD2-14;
TCB: cell culture supernatant of untransfected cells.

Claims

1. Polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens which include Rhesus D-specific CDR 1, CDR 2 and CDR 3 regions of pairs of amino acid sequences V_H and V_L with the same or different identification numbers according to the figures given in the table below:

	V_H				V_L			
Identification No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.
LD1-40	Fig. 1a	91-105	148-198	295-342	Fig. 1b	64-96	142-162	259-288
LD1-52	Fig. 2a	91-105	148-198	295-342	Fig. 2b	64-96	142-162	259-288
LD1-84	Fig. 3a	91-105	148-198	295-342	Fig. 3b	64-96	142-162	259-285
LD1-110	Fig. 4a	91-105	148-198	295-342	Fig. 4b	64-96	142-162	259-285
LD1-117	Fig. 5a	91-105	148-198	295-345	Fig. 5b	64-96	142-162	259-288
LD2-1	Fig. 6a	91-105	148-198	295-342	Fig. 6b	61-99	145-165	262-294
LD2-4	Fig. 7a	91-105	148-198	295-342	Fig. 7b	64-96	142-162	259-282
LD2-5	Fig. 8a	91-105	148-198	295-342	Fig. 8b	64-96	142-162	259-288
LD2-10	Fig. 9a	91-105	148-198	298-345	Fig. 9b	61-102	148-168	265-294
LD2-11	Fig. 10a	91-105	148-198	295-342	Fig. 10b	64-96	142-162	259-285
LD2-14	Fig. 11a	91-105	148-198	295-342	Fig. 11b	64-96	142-162	259-285
LD2-17	Fig. 12a	91-105	148-198	295-342	Fig. 12b	64-96	142-162	259-285
LD2-20	Fig. 13a	91-105	148-198	295-342	Fig. 13b	64-96	142-162	259-285
LD1-6-17	Fig. 14a	91-105	148-198	295-351	Fig. 14b	64-96	142-162	259-285
LD1/2-6-3	Fig. 15a	91-105	148-198	295-342	Fig. 15b	64-96	142-162	259-285
LD1/2-6-33	Fig. 16a	91-105	148-198	295-342	Fig. 16b	64-96	142-162	259-285

2. Polypeptides according to claim 1 which include Rhesus D-specific CDR 1, CDR 2 and CDR 3 regions of pairs of amino acid sequences V_H and V_L with the same identification numbers according to the figures given in the table of claim 1.

3. Polypeptides according to claim 1 which include regions with the amino acid sequences V_H and V_L and have identification numbers according to the figures given in the table of claim 1.

4. Polypeptides according to claim 1, 2 or 3 characterised as antigen binding Fab fragments.

5. Polypeptides according to claim 1, 2 or 3 comprising immunoglobulin heavy and light chains capable of forming complete anti-Rhesus D antibodies.

6. DNA sequences coding for polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens which include regions with the Rhesus D-specific CDR 1, CDR 2 and CDR 3 segments of pairs of DNA sequences V_H and V_L with the same or different identification numbers according to the figures given in the table below and functional equivalents thereof:

	V_H				V_L			
Identification No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.
LD1-40	Fig. 1a	91-105	148-198	295-342	Fig. 1b	64-96	142-162	259-288
LD1-52	Fig. 2a	91-105	148-198	295-342	Fig. 2b	64-96	142-162	259-288
LD1-84	Fig. 3a	91-105	148-198	295-342	Fig. 3b	64-96	142-162	259-285
LD1-110	Fig. 4a	91-105	148-198	295-342	Fig. 4b	64-96	142-162	259-285
LD1-117	Fig. 5a	91-105	148-198	295-345	Fig. 5b	64-96	142-162	259-288
LD2-1	Fig. 6a	91-105	148-198	295-342	Fig. 6b	61-99	145-165	262-294
LD2-4	Fig. 7a	91-105	148-198	295-342	Fig. 7b	64-96	142-162	259-282
LD2-5	Fig. 8a	91-105	148-198	295-342	Fig. 8b	64-96	142-162	259-288
LD2-10	Fig. 9a	91-105	148-198	298-345	Fig. 9b	61-102	148-168	265-294
LD2-11	Fig. 10a	91-105	148-198	295-342	Fig. 10b	64-96	142-162	259-285
LD2-14	Fig. 11a	91-105	148-198	295-342	Fig. 11b	64-96	142-162	259-285
LD2-17	Fig. 12a	91-105	148-198	295-342	Fig. 12b	64-96	142-162	259-285
LD2-20	Fig. 13a	91-105	148-198	295-342	Fig. 13b	64-96	142-162	259-285
LD1-6-17	Fig. 14a	91-105	148-198	295-351	Fig. 14b	64-96	142-162	259-285
LD1/2-6-3	Fig. 15a	91-105	148-198	295-342	Fig. 15b	64-96	142-162	259-285
LD1/2-6-33	Fig. 16a	91-105	148-198	295-342	Fig. 16b	64-96	142-162	259-285

7. DNA sequences according to claim 6 coding for polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens which include regions with the Rhesus D-specific CDR 1, CDR 2 and CDR 3 segments of pairs of DNA sequences V_H and V_L with the same

identification numbers according to the figures given in claim 6, and functional equivalents thereof.

8. DNA sequences according to claim 6 or 7 which include regions with the DNA sequences V_H and V_L with the identification numbers according to the figures given in claim 6.

9. DNA sequences according to claim 6, 7 or 8 coding for polypeptides capable of forming antigen binding Fab fragments.

10. DNA sequences according to claim 6, 7 or 8 coding for polypeptides capable of forming complete anti-Rhesus D antibodies.

11. A process for preparing recombinant polypeptides capable of forming antigen binding structures, e.g. Fab fragments, with specificity for Rhesus D antigens which process comprises the following steps in sequential order:

a) boosting of an individual capable of forming anti-Rhesus D antibodies with Rhesus D positive red blood cells,

b) isolating mononuclear cells from the individual,

c) isolating total RNA from the mononuclear cells,

d) preparing a cDNA by using an oligo(dT)primer and reverse transcribing of the mRNA with M-MuLV reverse transcriptase and amplifying the cDNA repertoire by a polymerase chain reaction using immunoglobulin gene family specific primers,

e) creating a phage display library by inserting the DNA coding for the heavy and light chain of the Fab polypeptide into a phagemid vector; the DNA for the heavy chain is inserted in frame to the gene coding for the phage protein pIII which allows the expression of a Fab pIII fusion protein on the surface of the phage,

f) transforming bacterial cells with the obtained recombinant plasmids, cultivating of the transformed bacterial cells and co-expression of the heavy and the light chain of a Fab on filamentous phage particles,

g) amplifying the Fab-carrying phage in bacteria,

h) selecting individual phage clones by several rounds of panning on Rhesus positive red blood cells.

i) isolating the plasmid DNA from the selected clones and cutting out the gIII gene,

j) transforming bacterial cells with the obtained plasmid, cultivating of the transformed bacterial cells expressing the Fab, and isolating the Fab fragments.

12. A process for selecting recombinant polypeptides capable of forming antigen binding structures with specificity for Rhesus D antigens and in particular showing reactivity with the Partial Rhesus DVI Variant and without any evidence of reactivity with red blood cells of Rhesus negative phenotypes in particular without reactivity against the Rhesus alleles C, c, E, and e which process comprises the following steps in sequential order:

a) performing several negative absorptions on the following red blood cells: phenotype 1 (r'r, Ccddee) treated with bromelase, phenotype 1 not treated with bromelase, phenotype 2 (ryry, CCddEE) treated with bromelase and phenotype 2 not treated with bromelase,

b) performing a positive absorption on DVI+ red blood cells with or without bromelase treatment,

c) determining the titer of phage binding to DVI+ red blood cells

d) repeating steps a), b) and c) until the titer of phage binding to DVI+ red blood cells has reached a satisfactory level.

13. A process according to claim 12, wherein the recombinant polypeptides capable of forming antigen binding structures are Fab fragments.

14. Anti-Rhesus D antibodies having heavy and light chain variable regions comprising the Rhesus D-specific CDR 1, CDR 2 and CDR 3

sequences of pairs of amino acid sequences V_H and V_L having the same or different identification numbers according to the table below:

	V_H				V_L			
Identification No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.	Figure	CDR 1 base pair No.	CDR 2 base pair No.	CDR 3 base pair No.
LD1-40	Fig. 1a	91-105	148-198	295-342	Fig. 1b	64-96	142-162	259-288
LD1-52	Fig. 2a	91-105	148-198	295-342	Fig. 2b	64-96	142-162	259-288
LD1-84	Fig. 3a	91-105	148-198	295-342	Fig. 3b	64-96	142-162	259-285
LD1-110	Fig. 4a	91-105	148-198	295-342	Fig. 4b	64-96	142-162	259-285
LD1-117	Fig. 5a	91-105	148-198	295-345	Fig. 5b	64-96	142-162	259-288
LD2-1	Fig. 6a	91-105	148-198	295-342	Fig. 6b	61-99	145-165	262-294
LD2-4	Fig. 7a	91-105	148-198	295-342	Fig. 7b	64-96	142-162	259-282
LD2-5	Fig. 8a	91-105	148-198	295-342	Fig. 8b	64-96	142-162	259-288
LD2-10	Fig. 9a	91-105	148-198	298-345	Fig. 9b	61-102	148-168	265-294
LD2-11	Fig. 10a	91-105	148-198	295-342	Fig. 10b	64-96	142-162	259-285
LD2-14	Fig. 11a	91-105	148-198	295-342	Fig. 11b	64-96	142-162	259-285
LD2-17	Fig. 12a	91-105	148-198	295-342	Fig. 12b	64-96	142-162	259-285
LD2-20	Fig. 13a	91-105	148-198	295-342	Fig. 13b	64-96	142-162	259-285
LD1-6-17	Fig. 14a	91-105	148-198	295-351	Fig. 14b	64-96	142-162	259-285
LD1/2-6-3	Fig. 15a	91-105	148-198	295-342	Fig. 15b	64-96	142-162	259-285
LD1/2-6-33	Fig. 16a	91-105	148-198	295-342	Fig. 16b	64-96	142-162	259-285

15. Anti-Rhesus D antibodies having heavy and light chain variable regions comprising the Rhesus D-specific CDR 1, CDR 2 and CDR 3 sequences of pairs of amino acid sequences V_H and V_L having the same identification numbers as indicated in the table of claim 14.

16. Anti-Rhesus D antibodies according to claim 14 or 15 which include pairs of amino acid sequences V_H and V_L having the identification numbers according to the figures, as indicated in the table of claim 14.

17. Anti-Rhesus D antibodies according to claims 14, 15 , or 16 wherein the immunoglobulin constant regions are of at least one of the defined isotypes IgG1, IgG2, IgG3 or IgG4.

18. A process for preparing complete anti-Rhesus D antibodies according to one of the claims 14 to 17, comprising in sequential order the steps of

a) amplifying separately the members of a pair of a heavy chain V gene segment and a light chain V gene segment containing Rhesus D-specific CDR 1, CDR 2 and CDR 3 regions as depicted in Figs. 1a - 16a and 1b - 16b, respectively, from an anti-Rhesus D-Fab-encoding plasmid by carrying out a polymerase chain reaction with specific primers,

b) preparing separately the genes of a complete anti-Rhesus D immunoglobulin heavy chain and a complete anti-Rhesus D immunoglobulin light chain in suitable plasmids containing the immunoglobulin constant region gene segments coding for either one of the human γ1, γ2, γ3 and γ4 heavy chains and for the human κ or λ light chain and transforming the obtained plasmids separately in suitable E. coli bacteria, and

c) cotransfecting the obtained plasmids into suitable eukaryotic host cells, cultivating of the cells, separating the non-transformed cells, cloning of the cultures, selecting the best producing clone, using it as a production culture and isolating the complete antibodies from the supernatant of the cell culture.

19. A pharmaceutical composition comprising at least one polypeptide according to the definition of claim 1, 2 or 3 or at least one anti-Rhesus D antibody according to one of the claims 14 to 17 for the prophylaxis of haemolytic disease of the newborn, for the treatment of idiopathic thrombocytopenic purpura and mistransfusions of Rhesus incompatible blood.

20. A diagnostic composition for Rhesus D typing comprising Fab fragments according to claim 4 or anti-Rhesus D antibodies according to one of the claims 14 to 17.

Fig. 1a

LD1-40-VH sequence

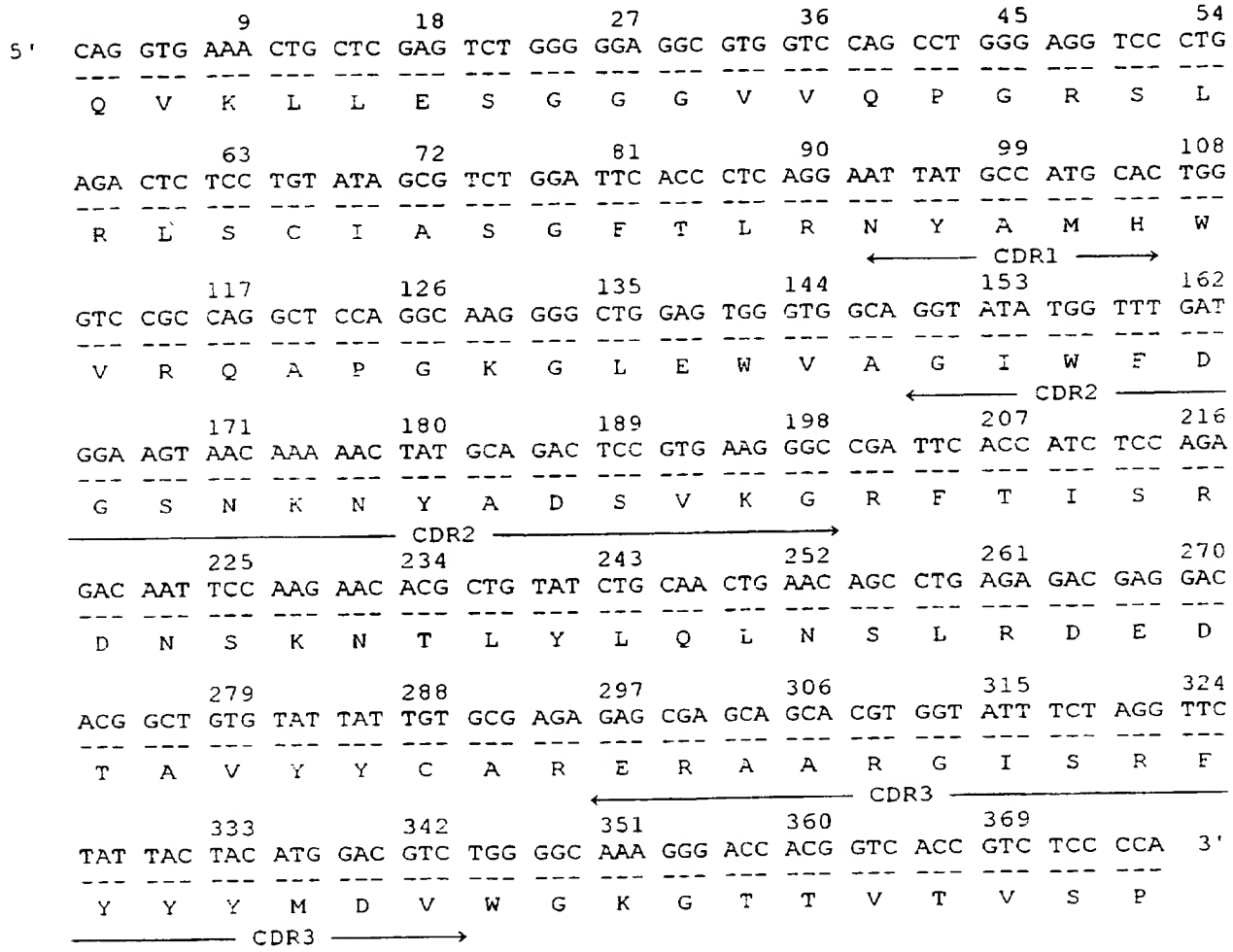


Fig. 1b

LD1-40-VL sequence

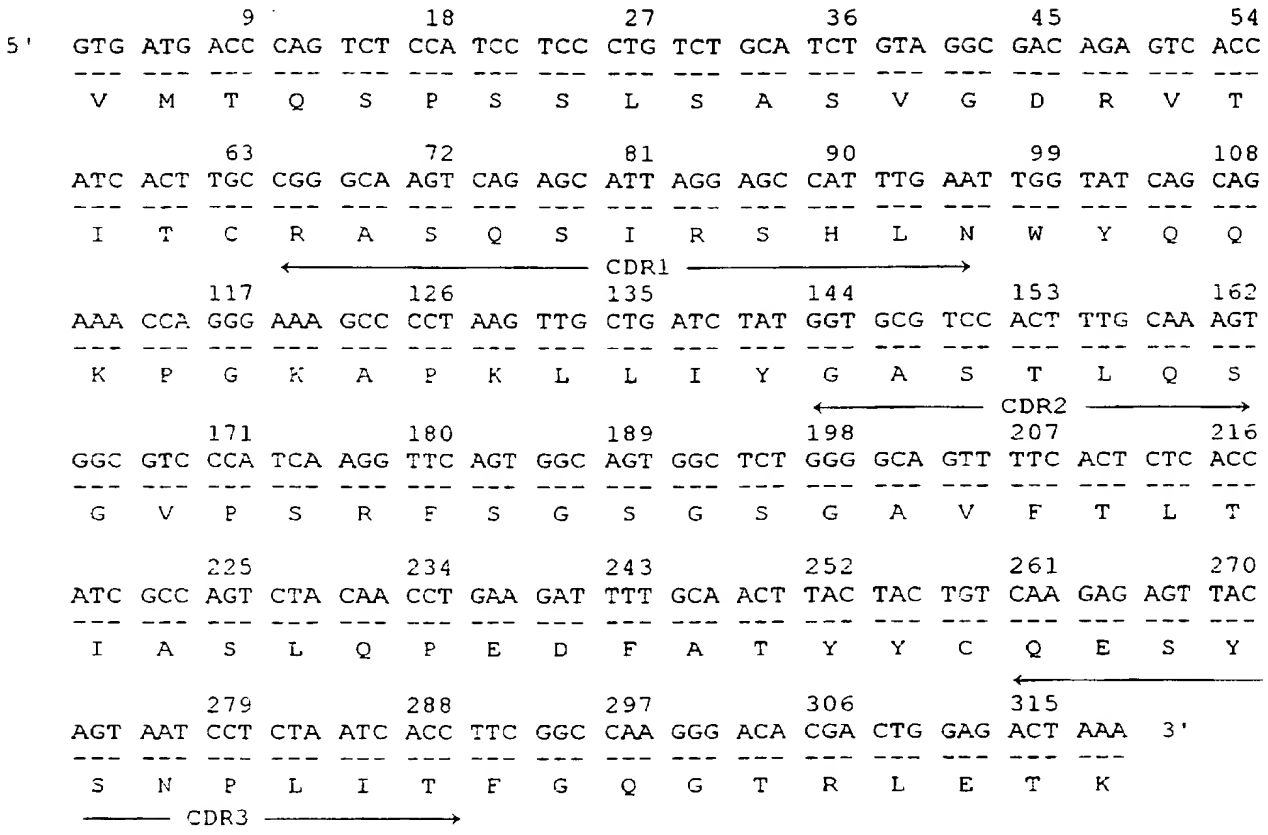


Fig. 2a

LD1-52-VH sequence

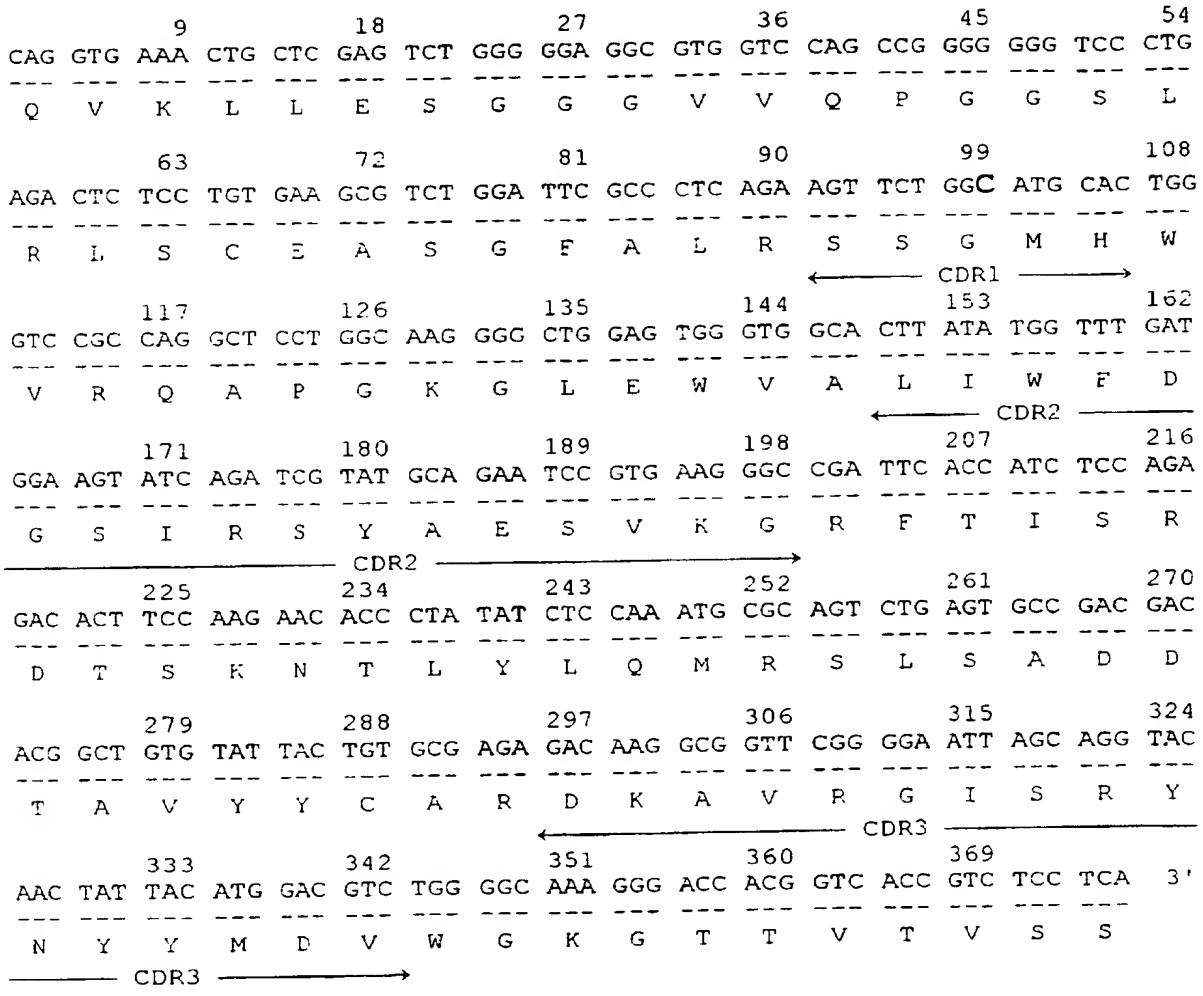


Fig. 2b

LD1-52-VL sequence

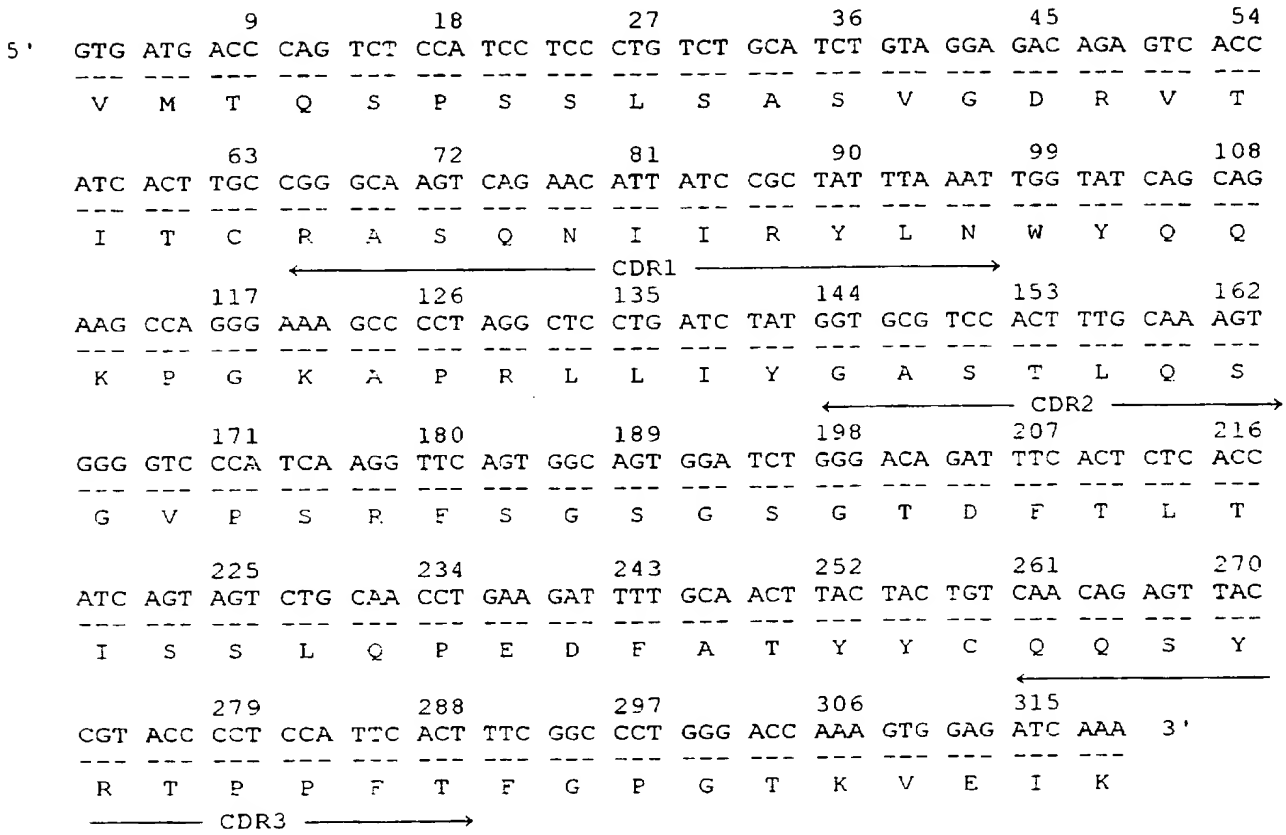


Fig. 3a

LD1-84-VH sequence

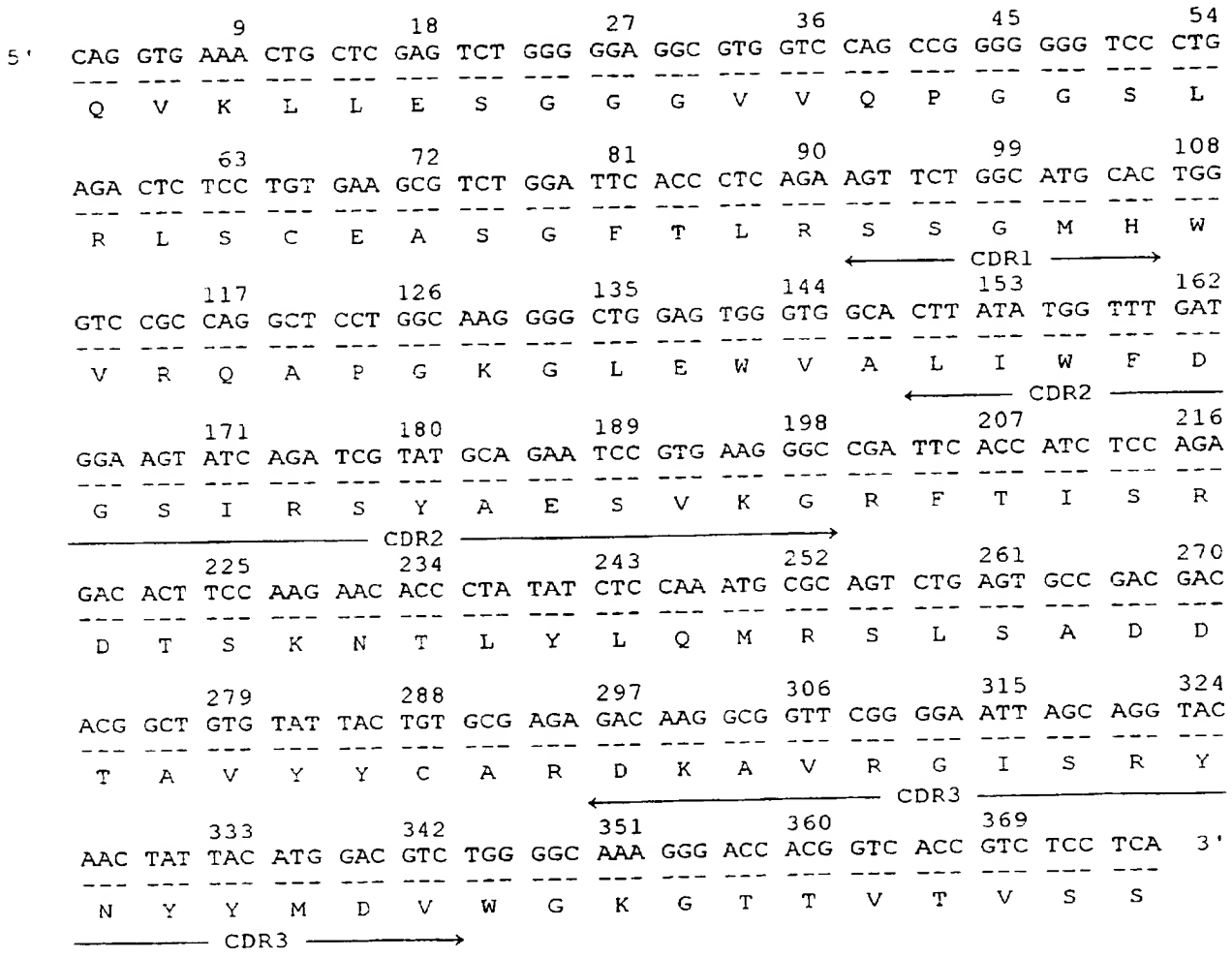


Fig. 3b

LD1-84-VL sequence

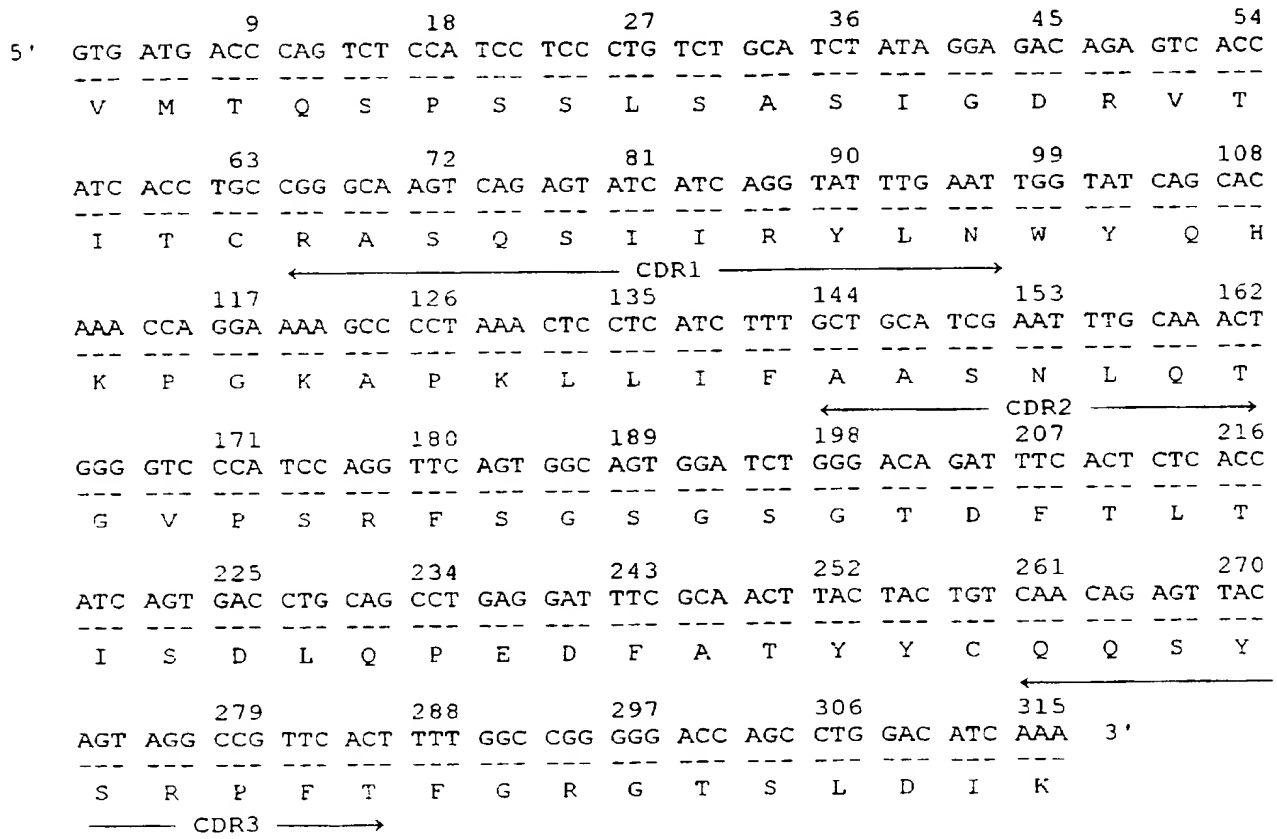


Fig. 4a

LD1-110-VH sequence

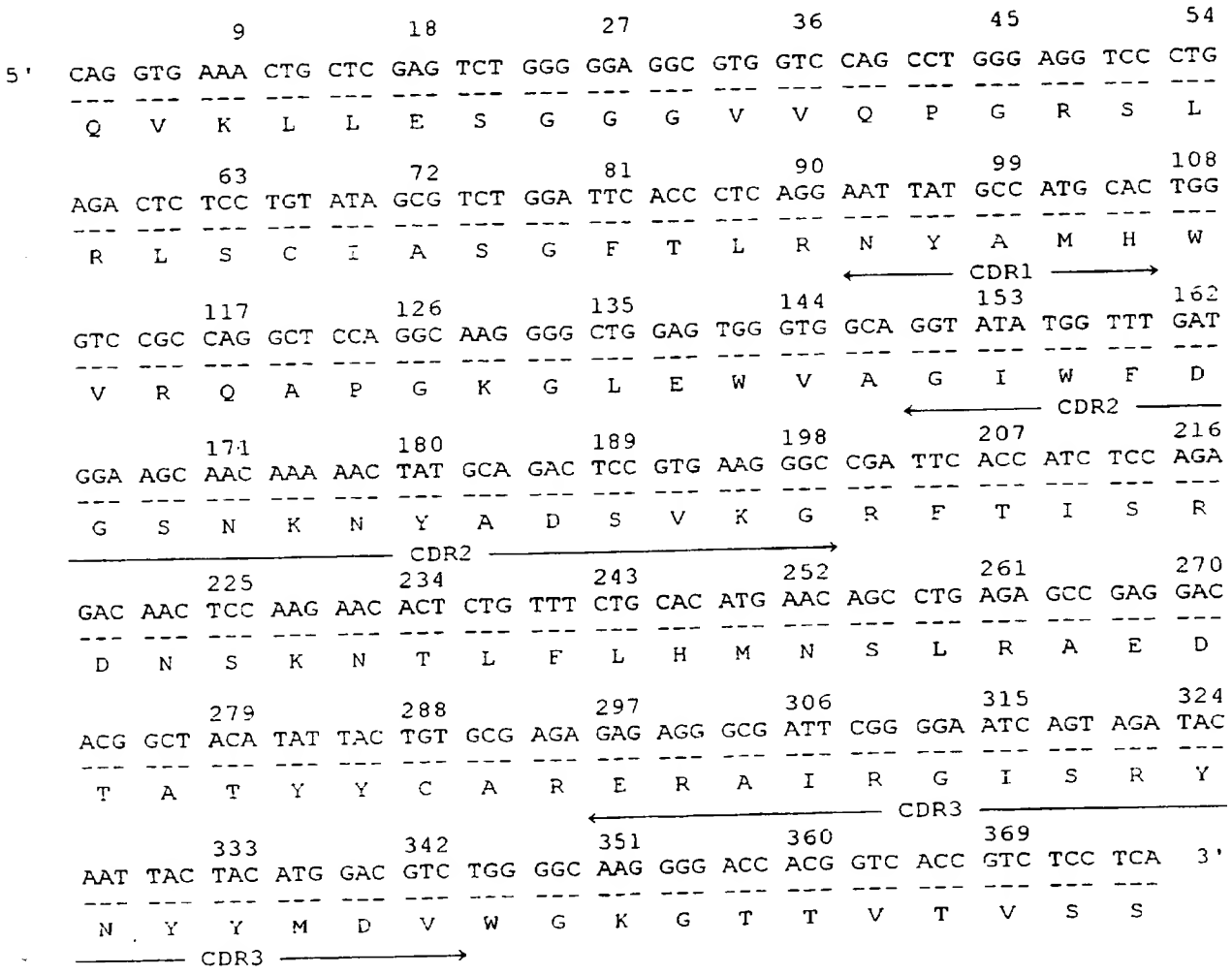


Fig. 4b

LD1-110-VL sequence

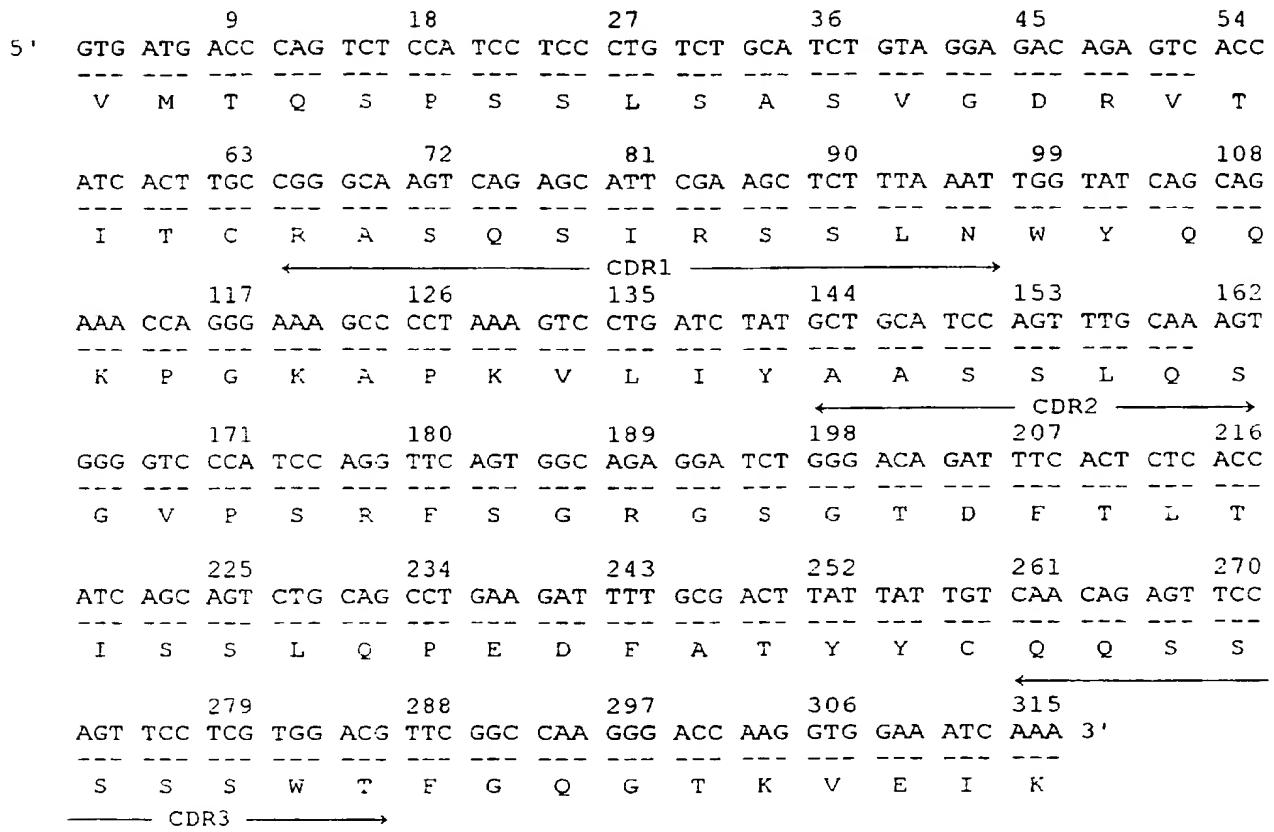


Fig. 5a

LD1-117-VH sequence

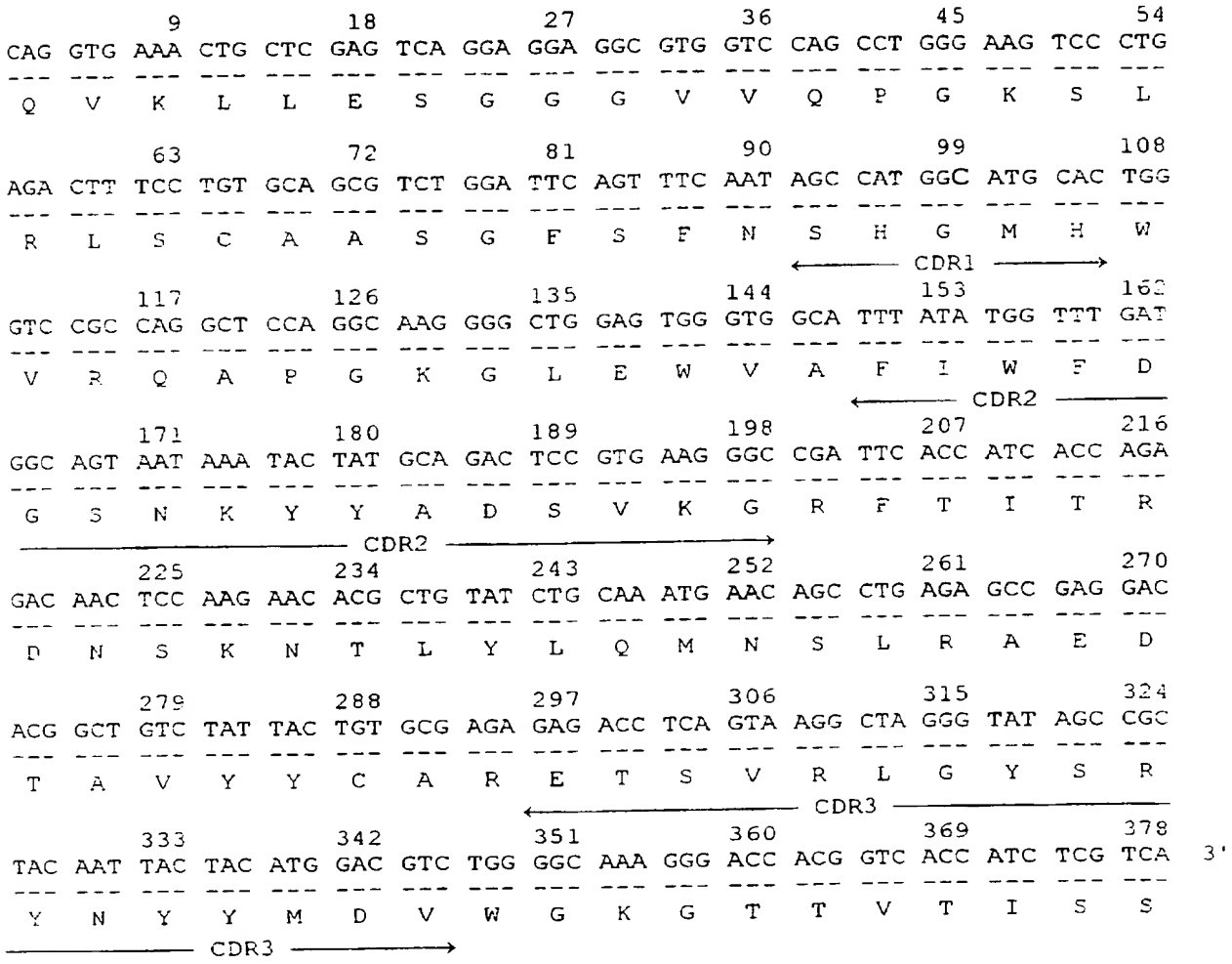


Fig. 5b

LD1-117-VL sequence

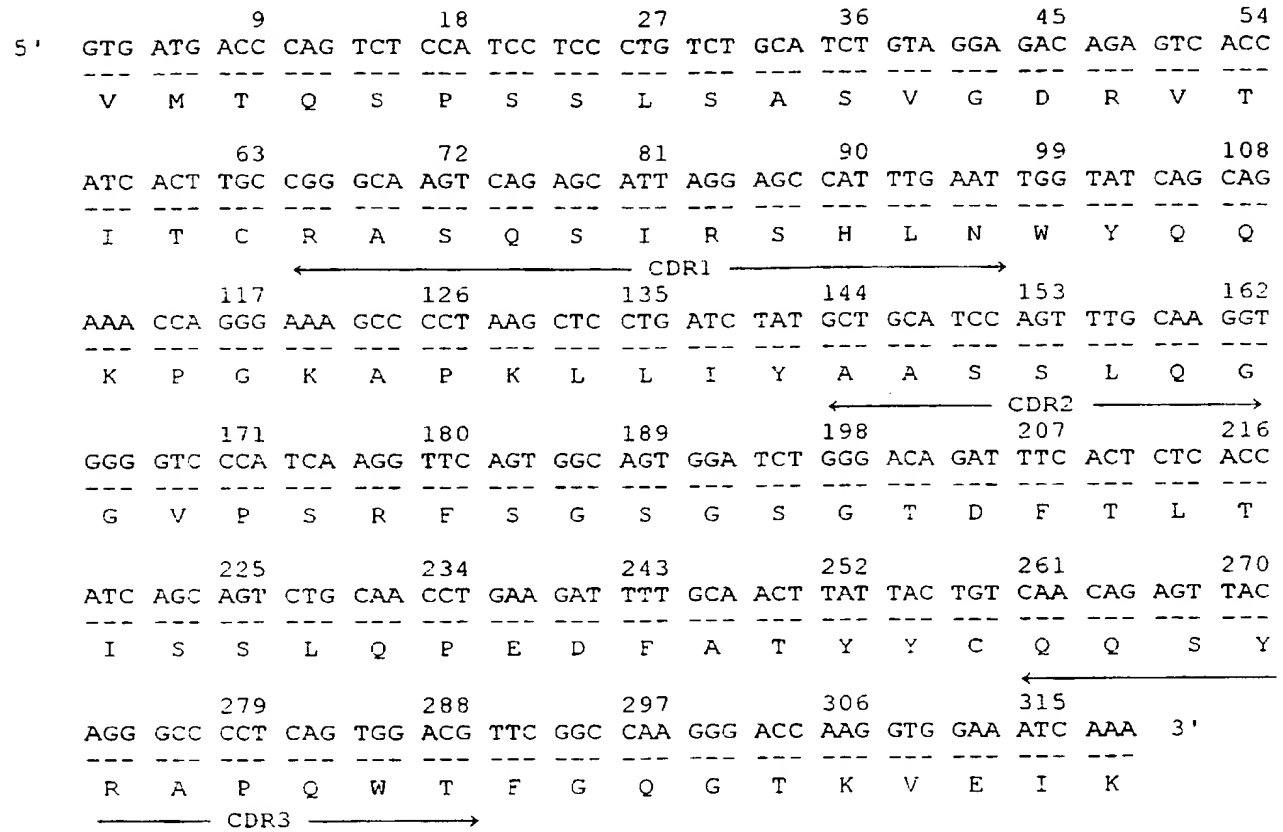


Fig. 6a

LD2-1-VH sequence

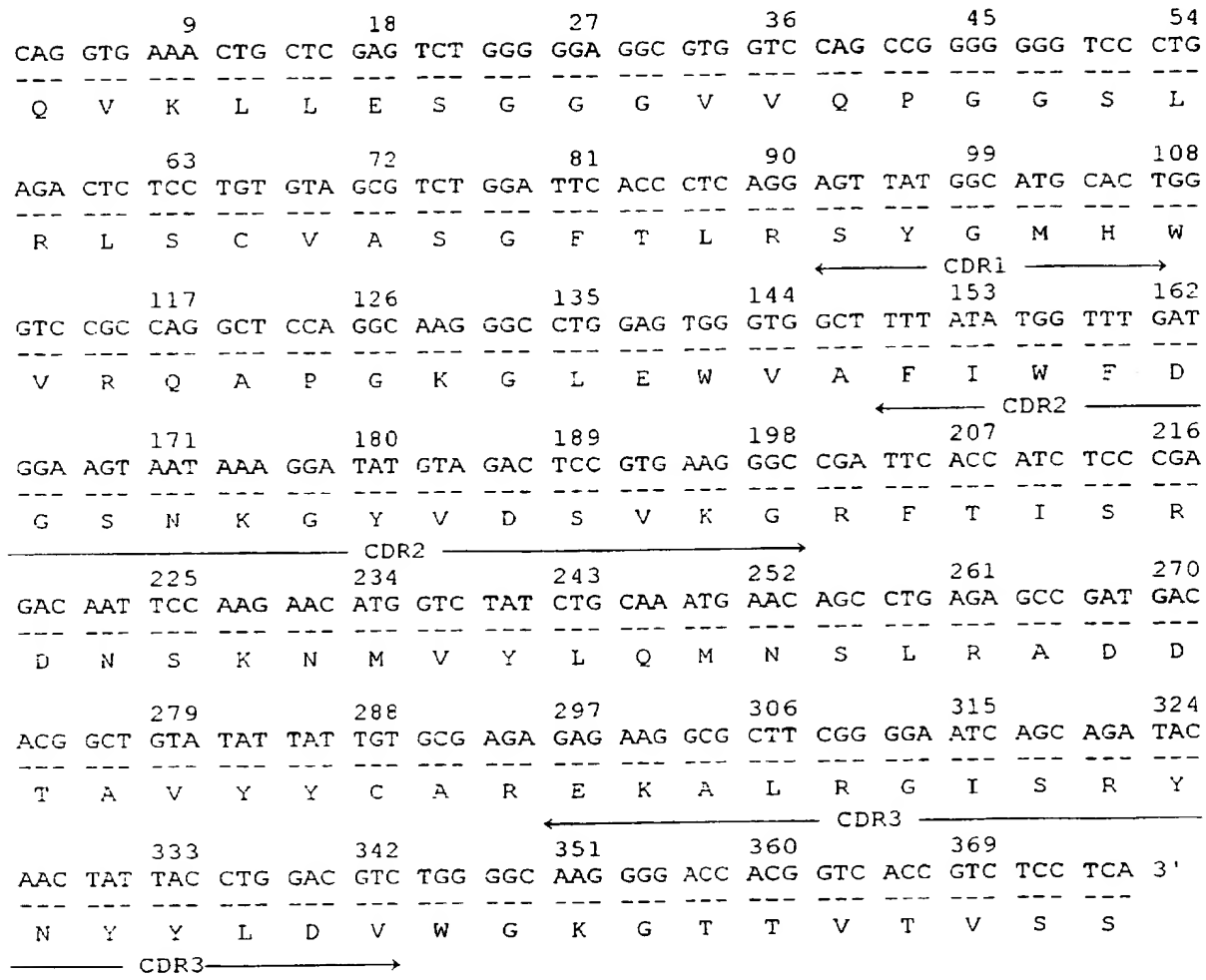


Fig. 6b

LD2-1-VL sequence

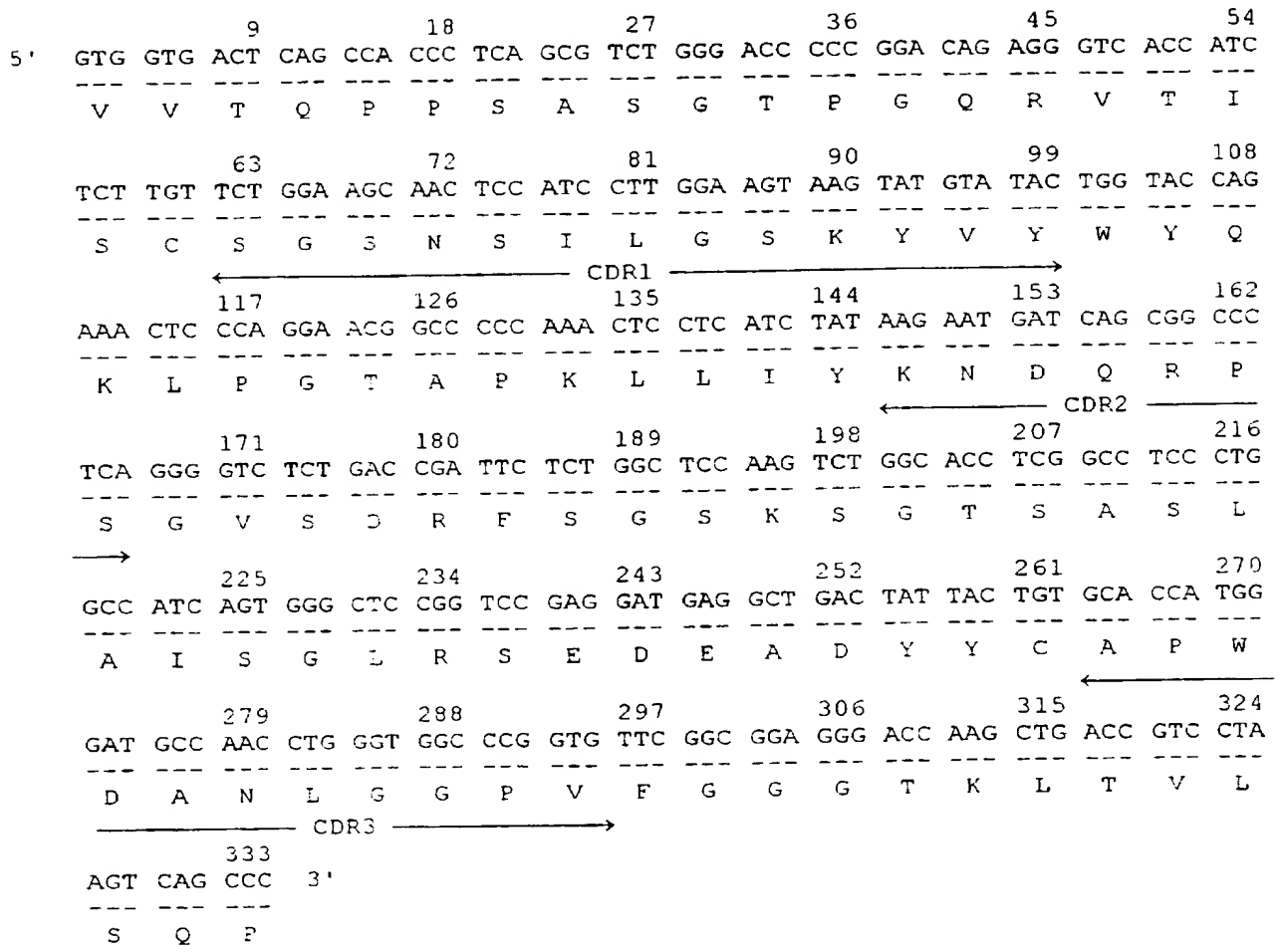


Fig. 7a

LD2-4-VH sequence

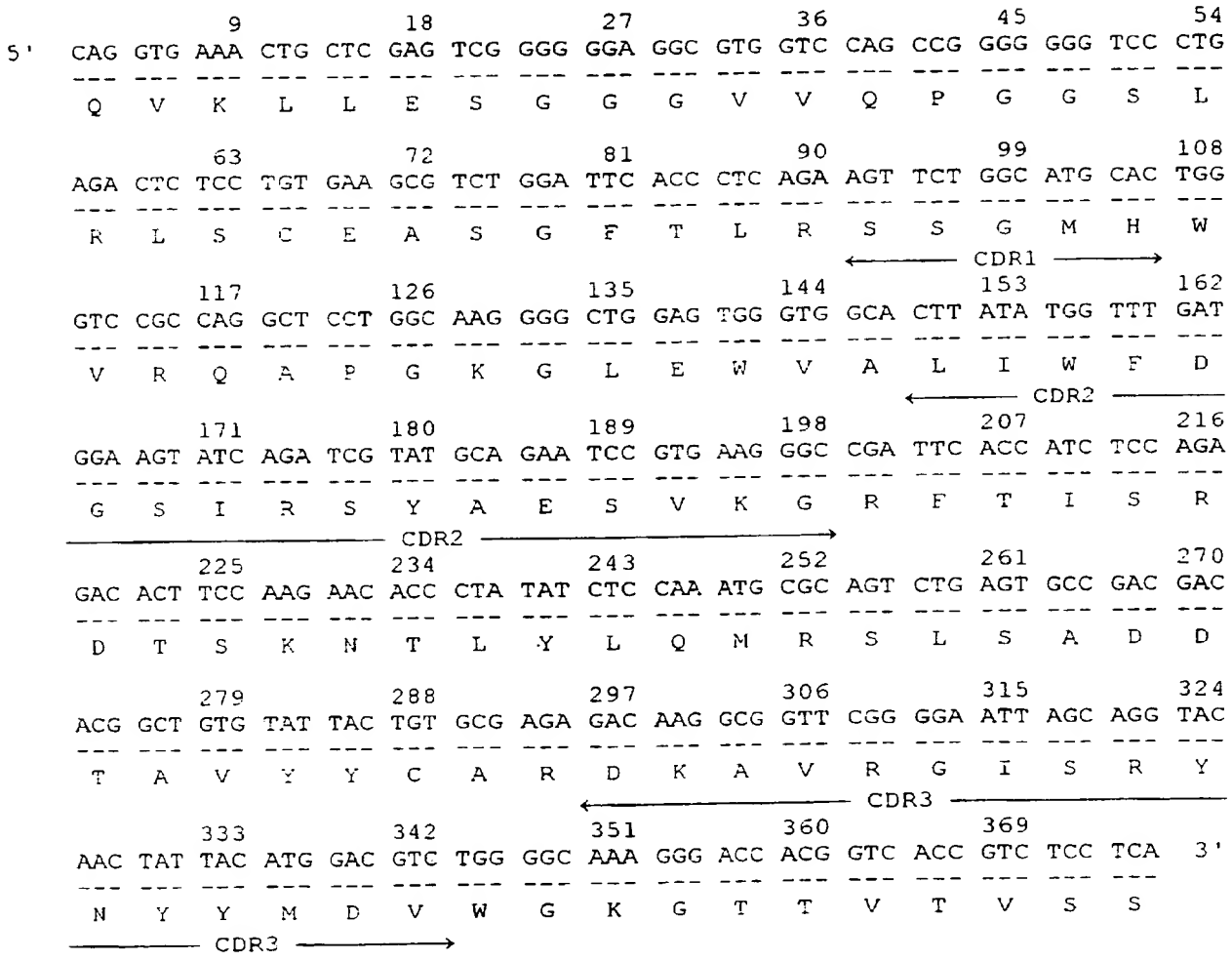


Fig. 7b

LD2-4-VL sequence

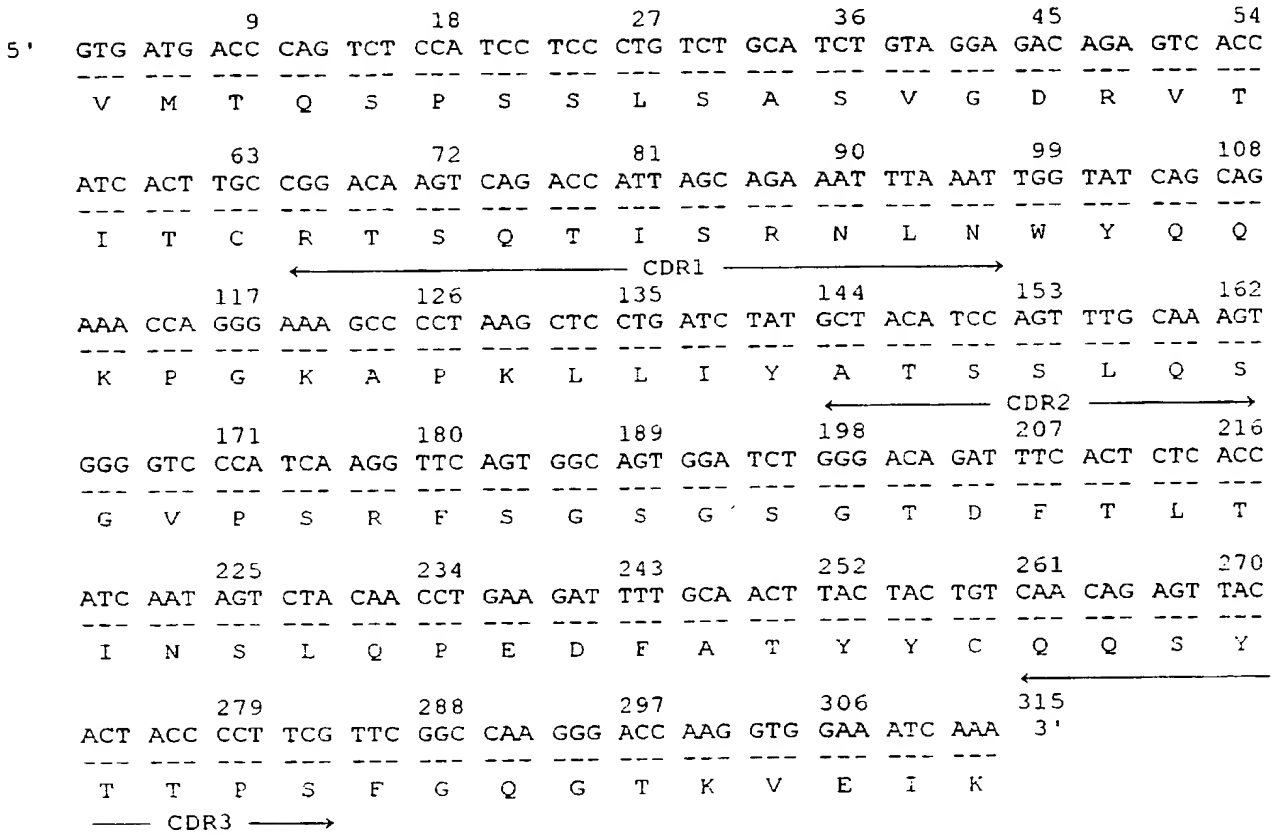


Fig. 8a

LD2-5-VH sequence

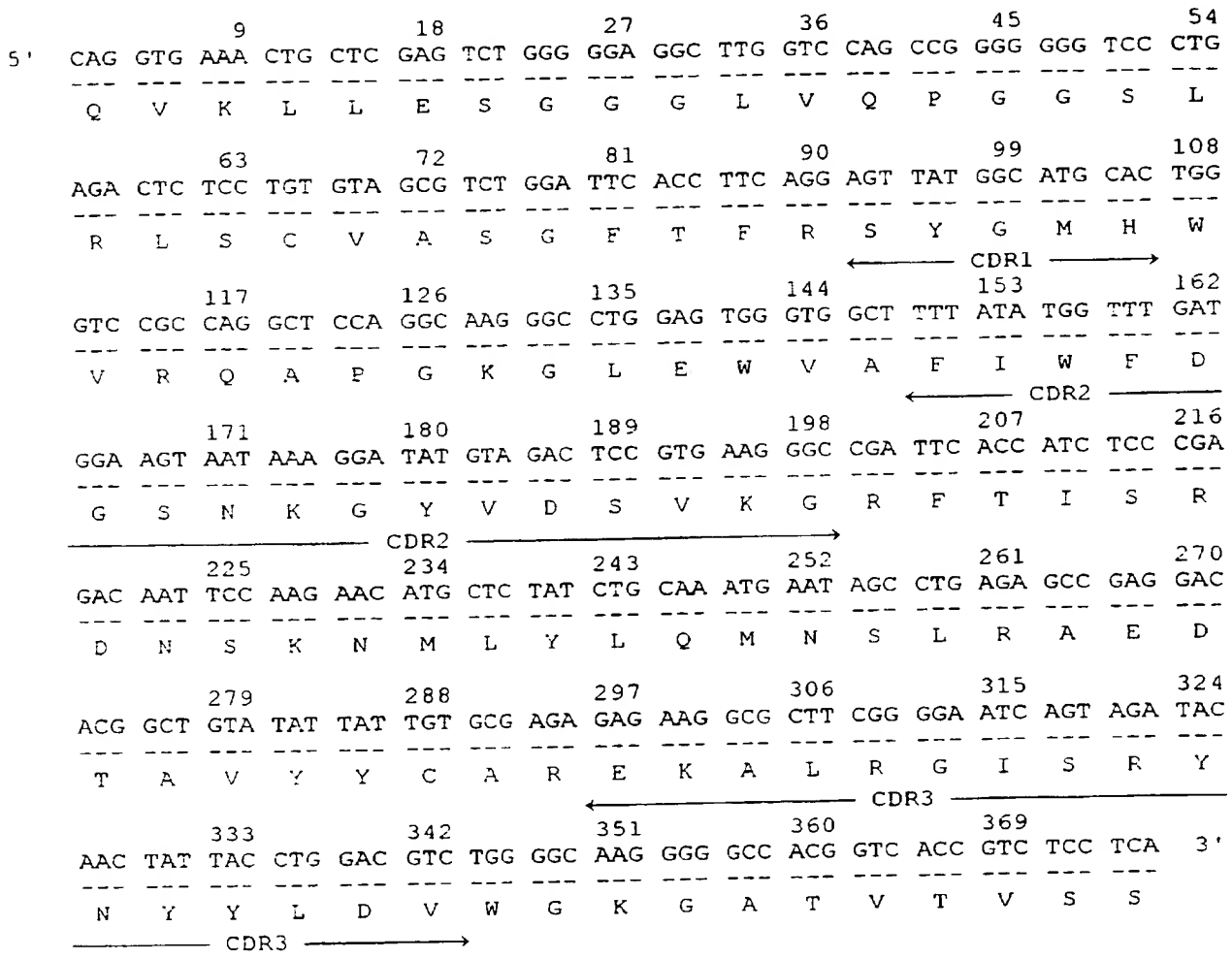


Fig. 8b

LD2-5-VL sequence

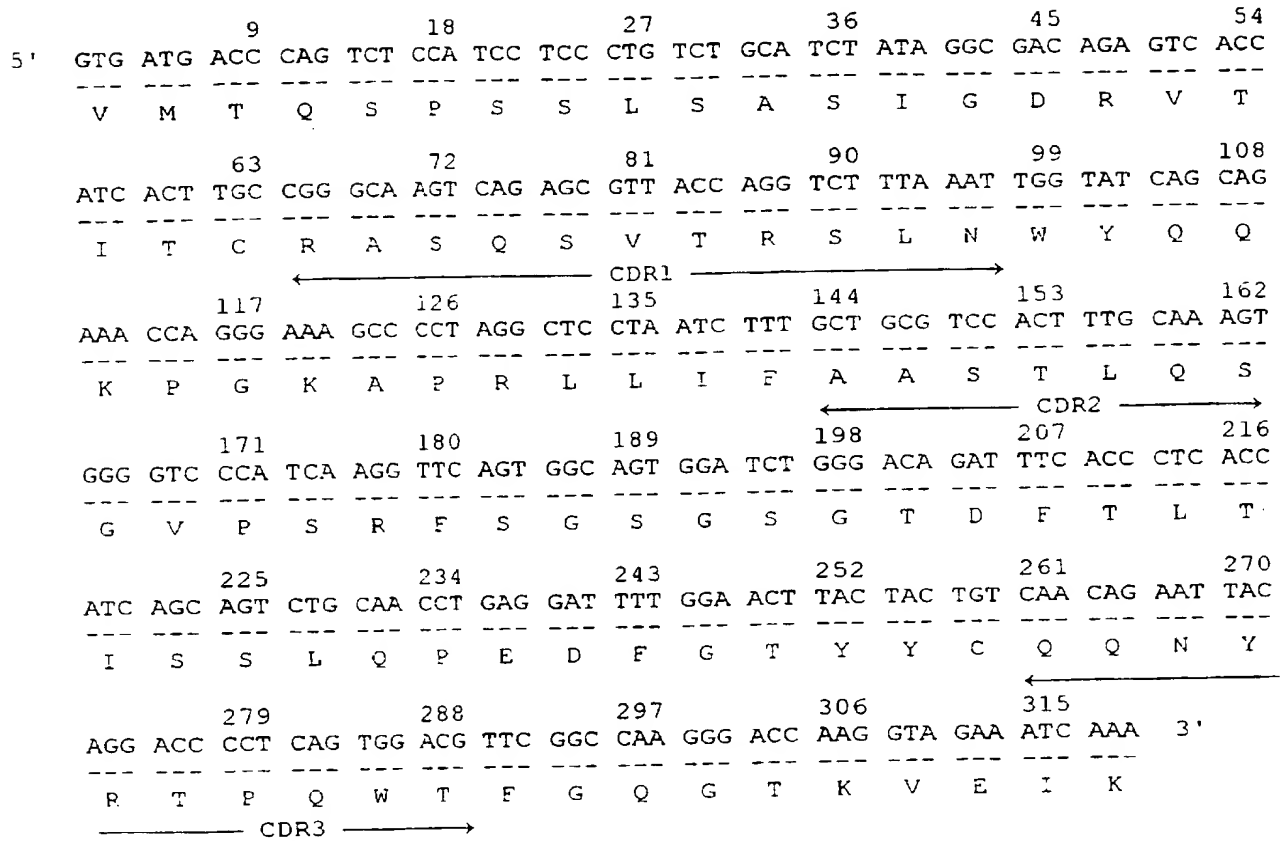


Fig. 9a

LD2-10-VH sequence

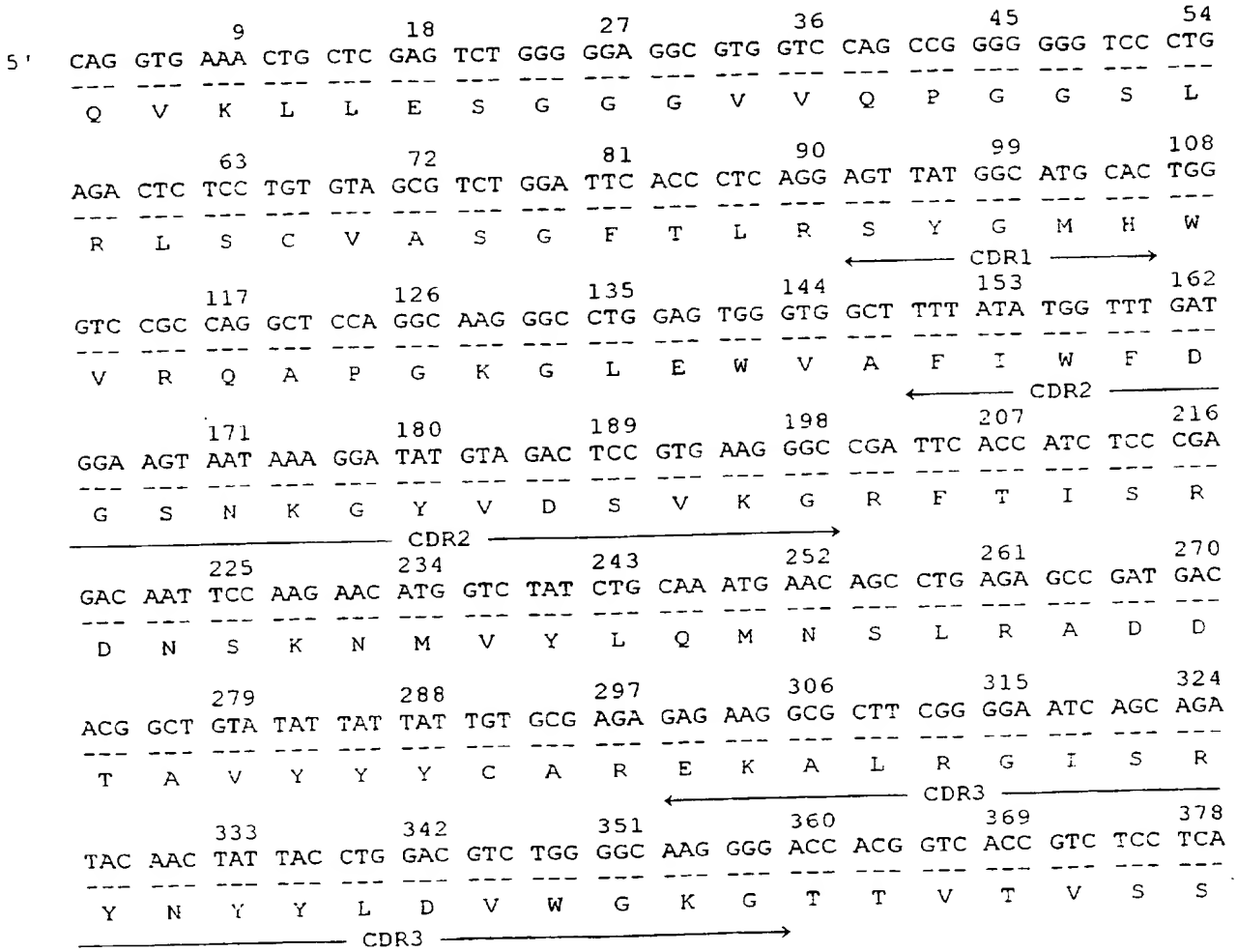


Fig. 9b

LD2-10-VL sequence

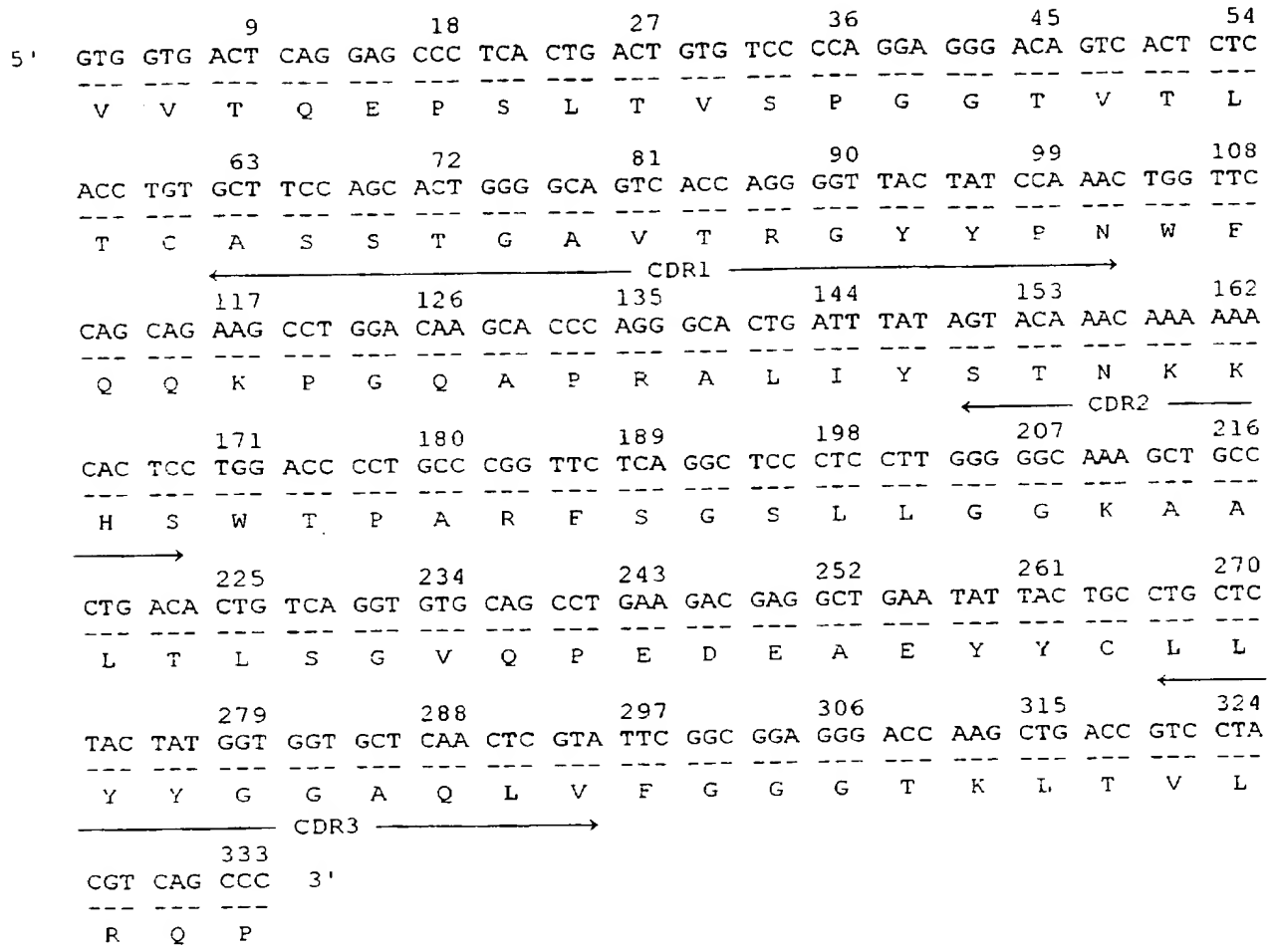


Fig. 10a

LD2-11-VH sequence

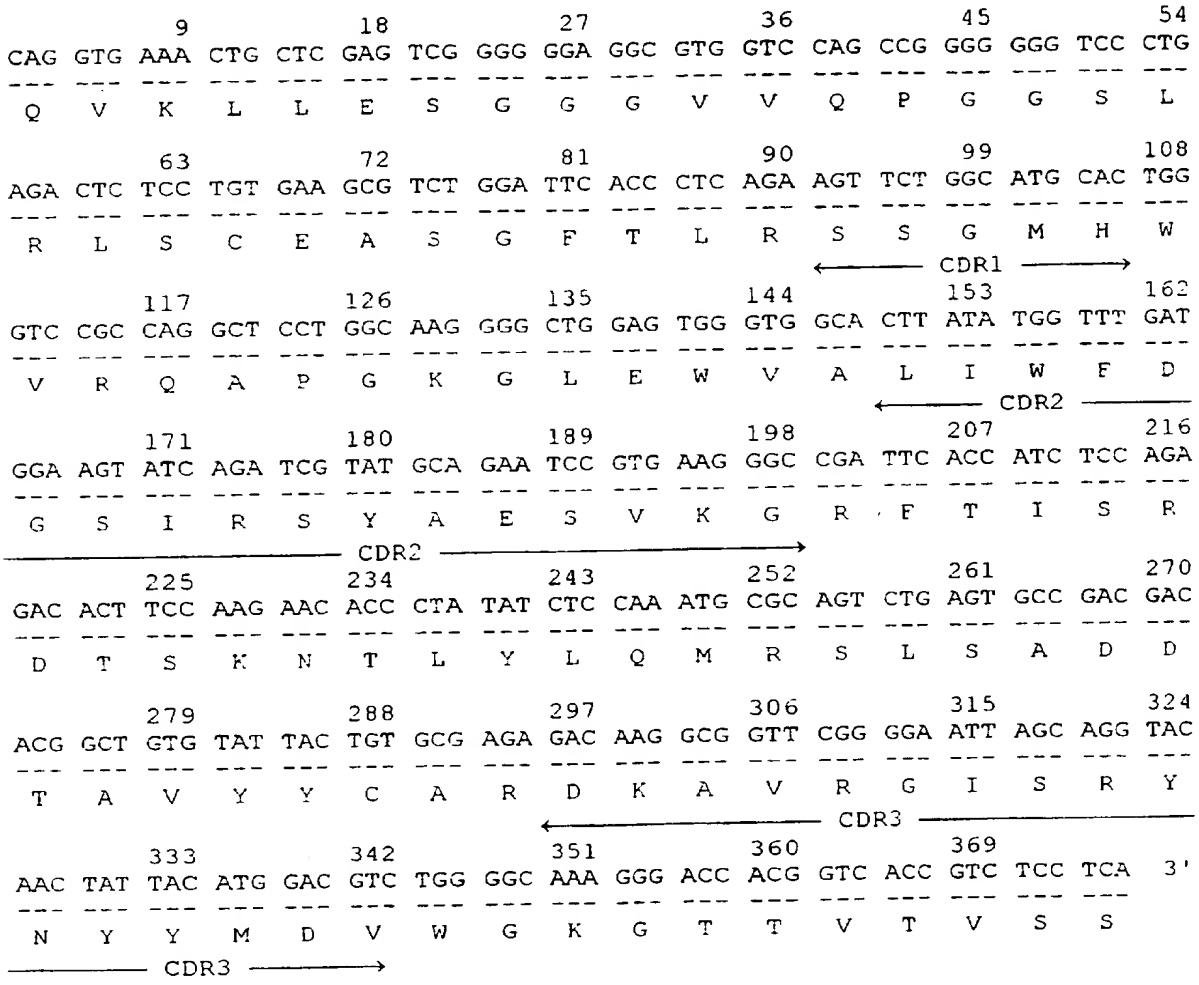


Fig. 10b

LD2-11-VL sequence

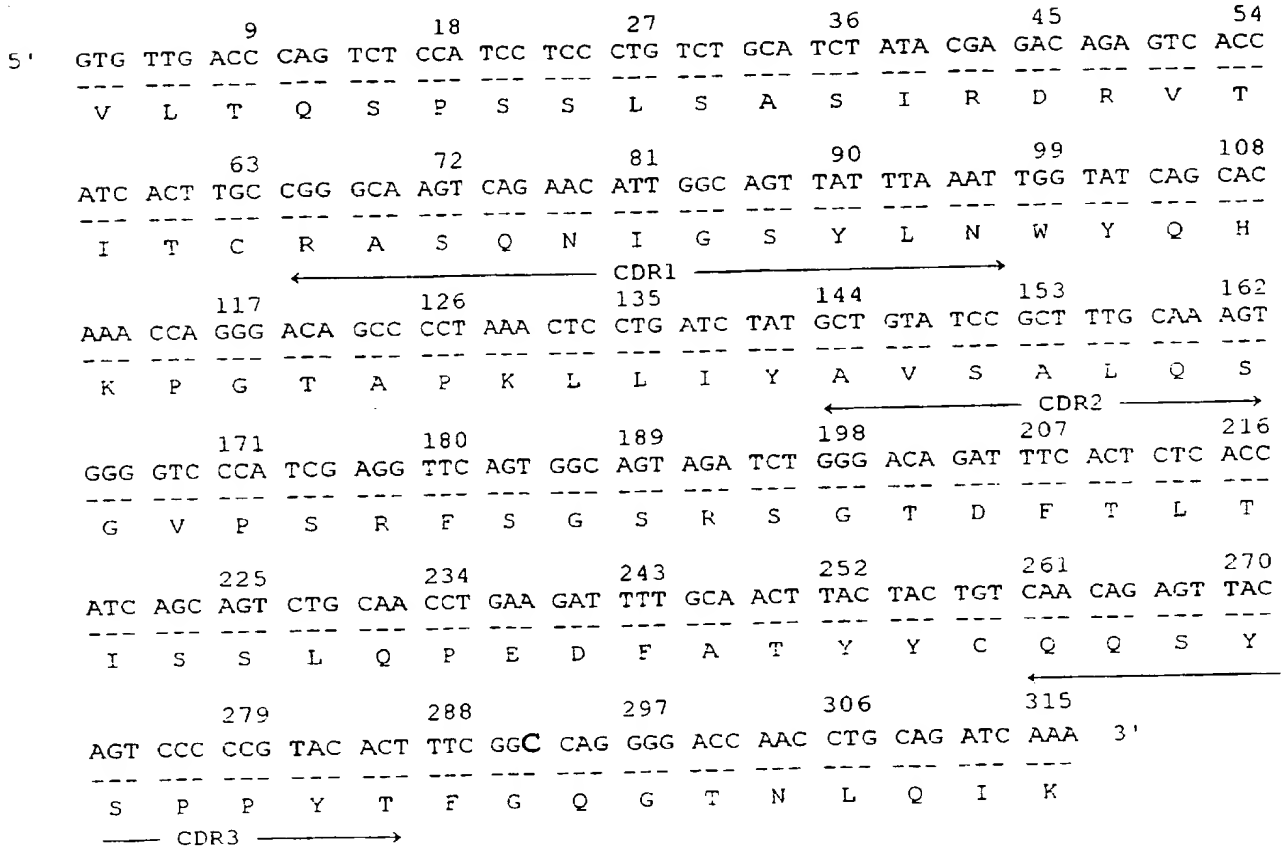


Fig. 11a

LD2-14-VH sequence

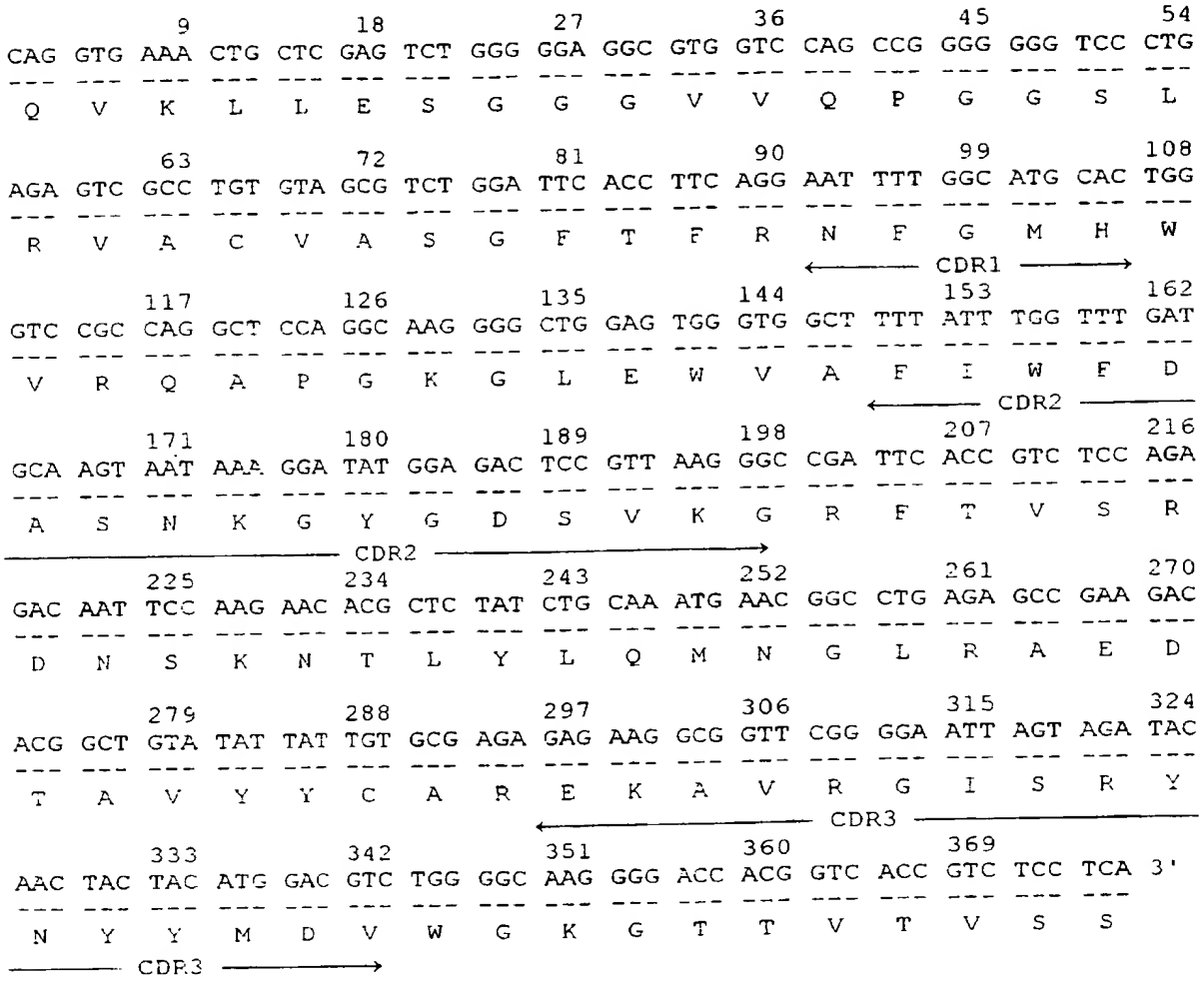


Fig. 11b

LD2-14-VL sequence

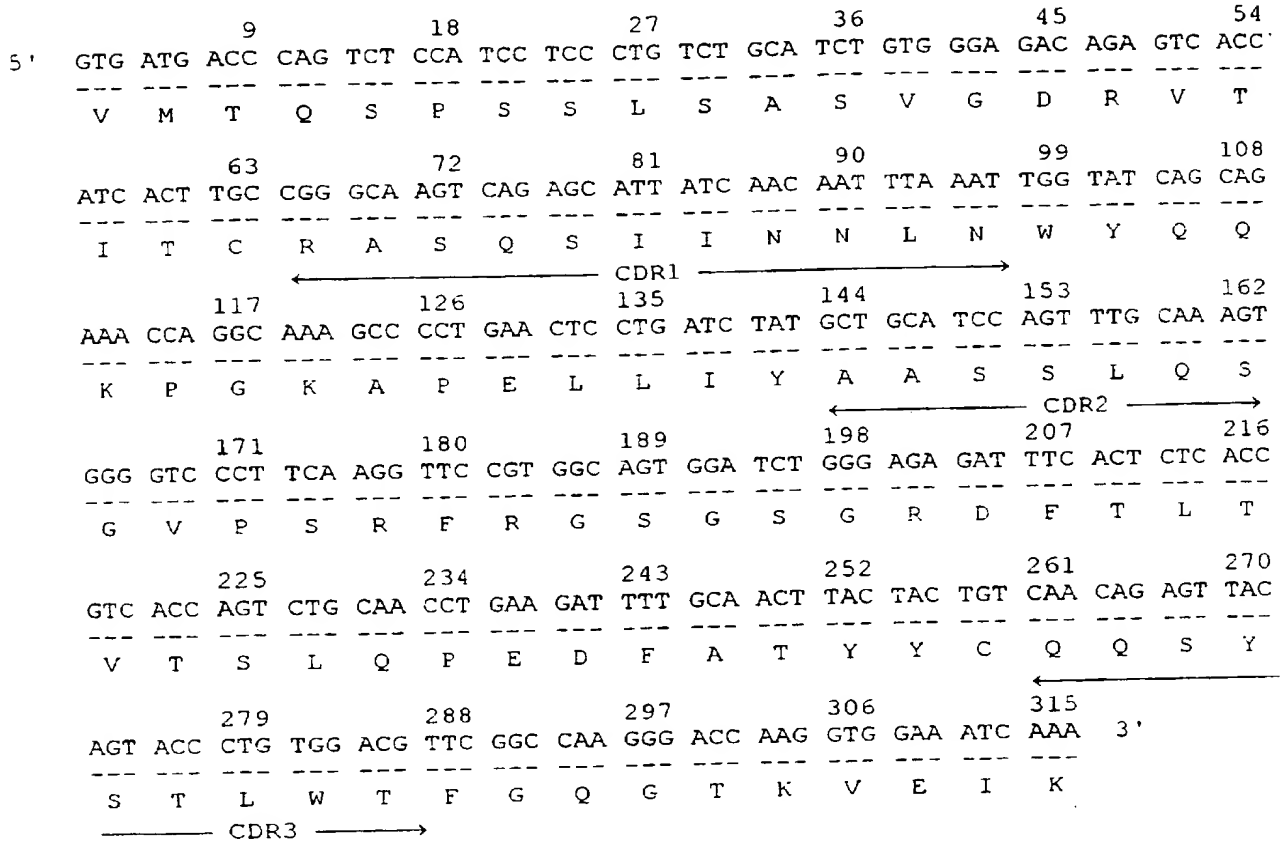


Fig. 12a

LD2-17-VH sequence

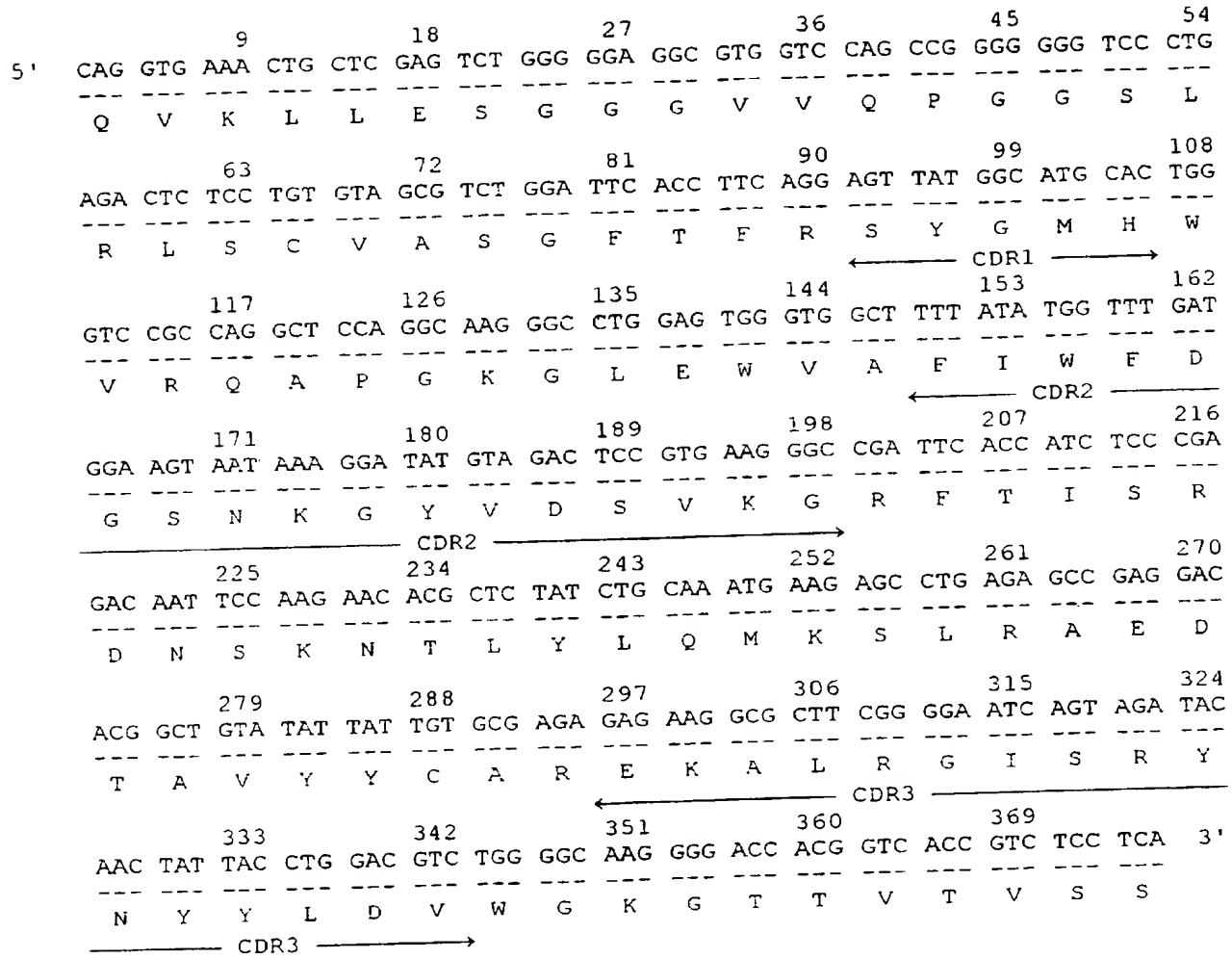


Fig. 12b

LD2-17-VL sequence

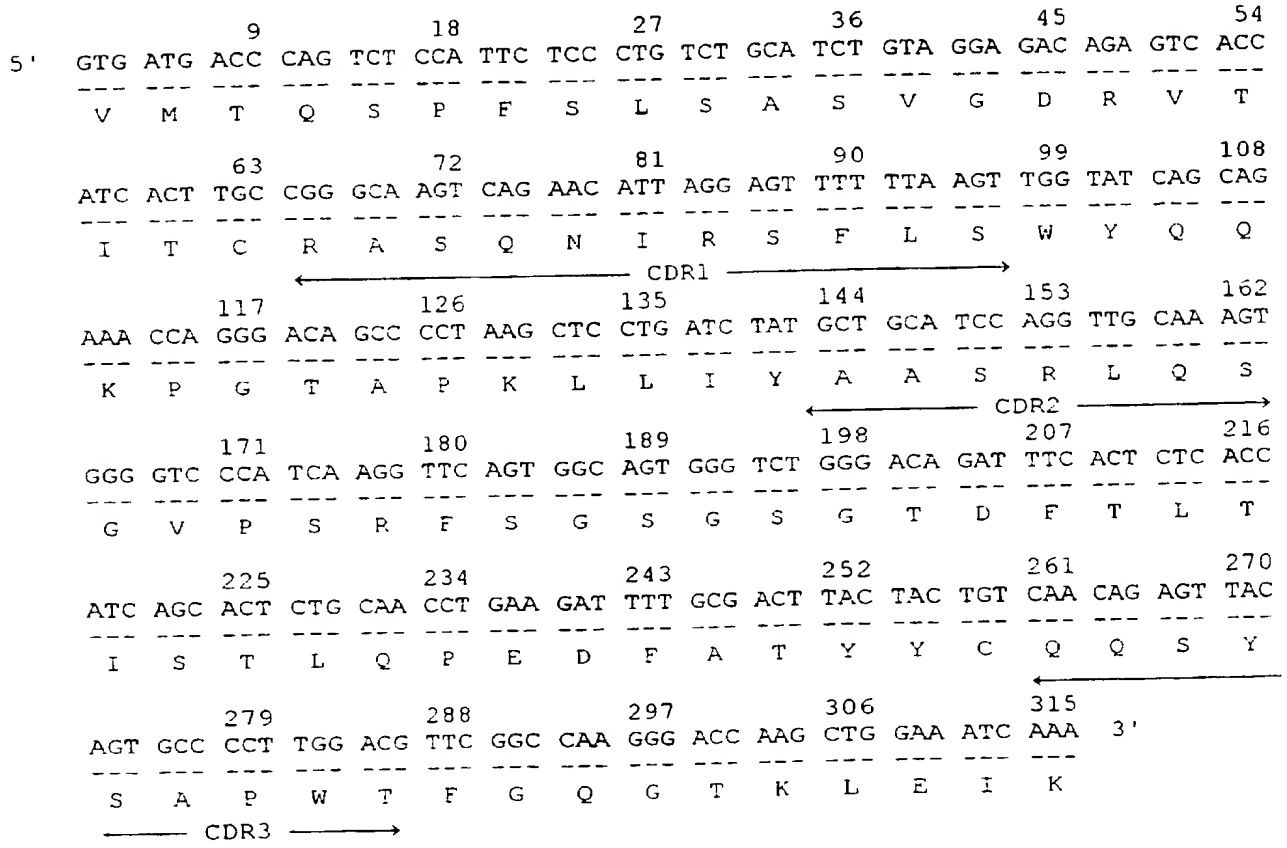


Fig. 13a

LD2-20-VH sequence

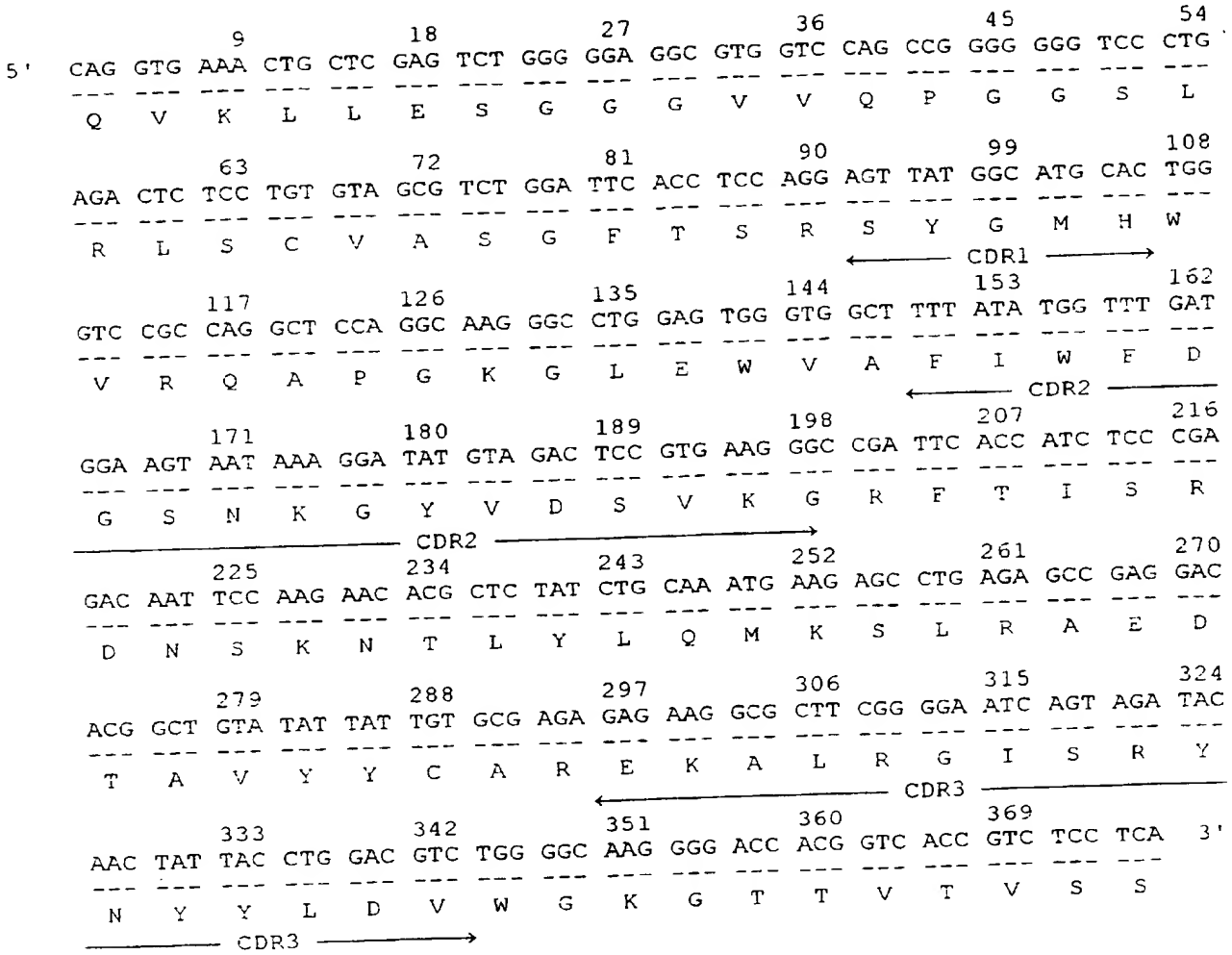


Fig. 13b

LD2-20-VL sequence

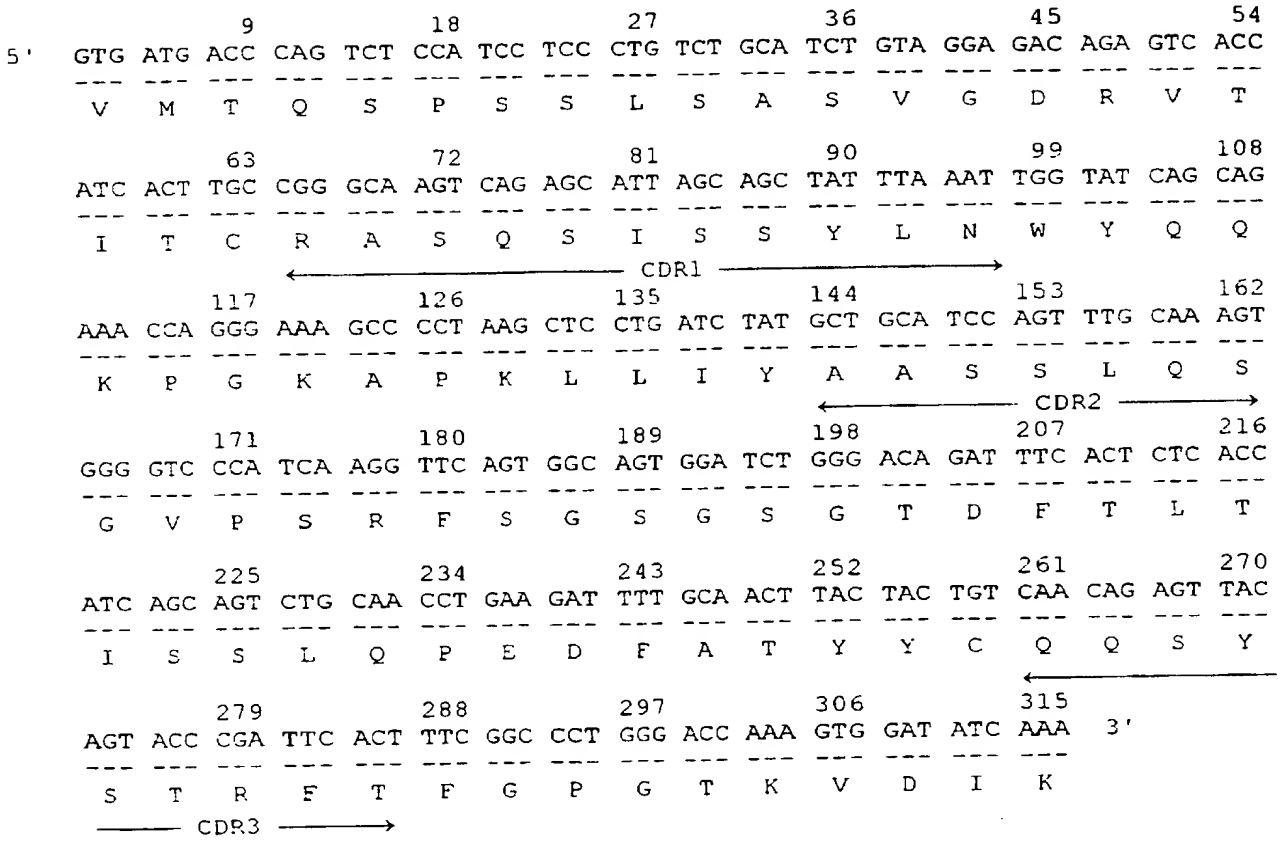


Fig. 14a

LD1-6-17-VH sequence

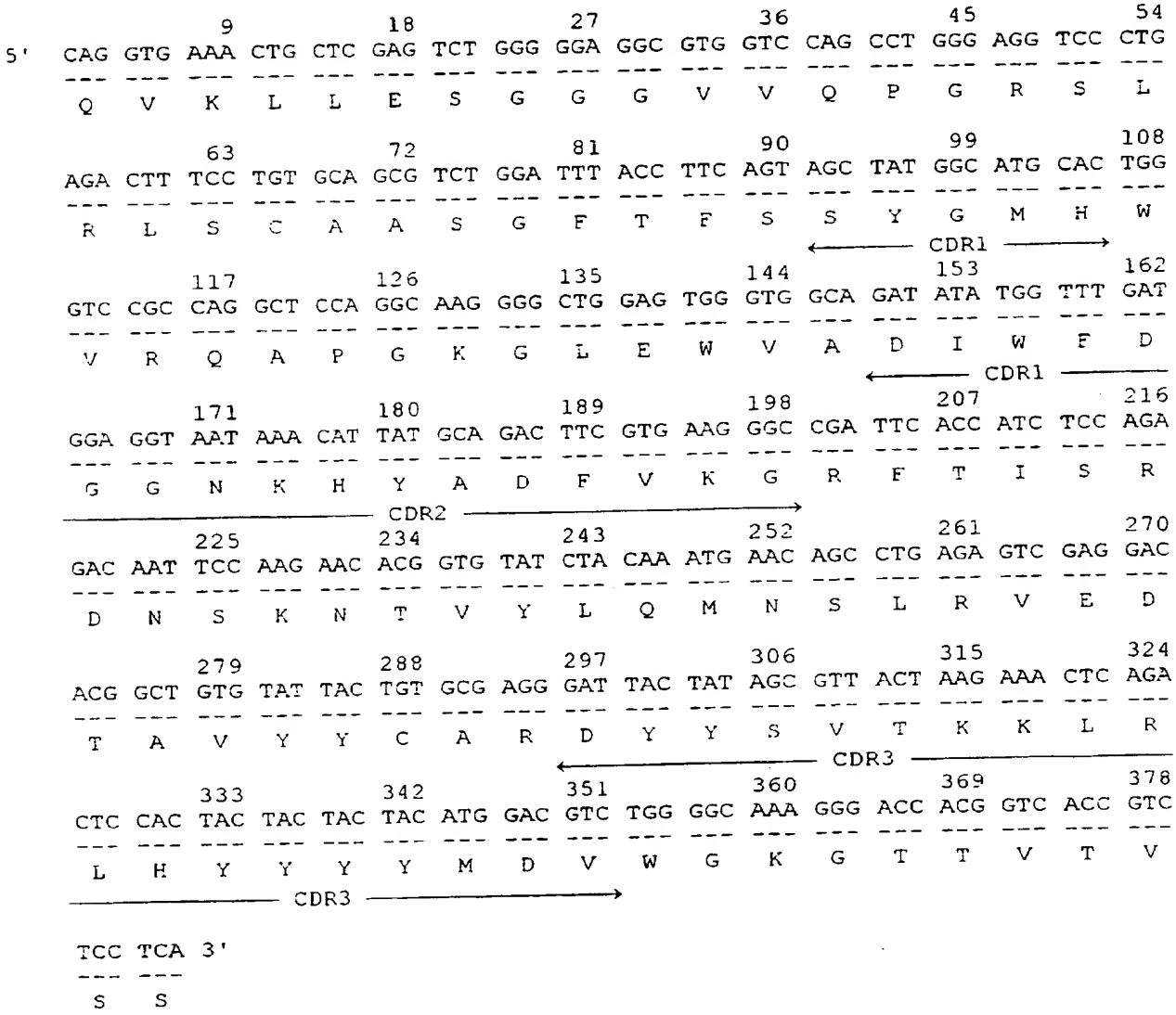


Fig. 14b

LD1-6-17-VL sequence

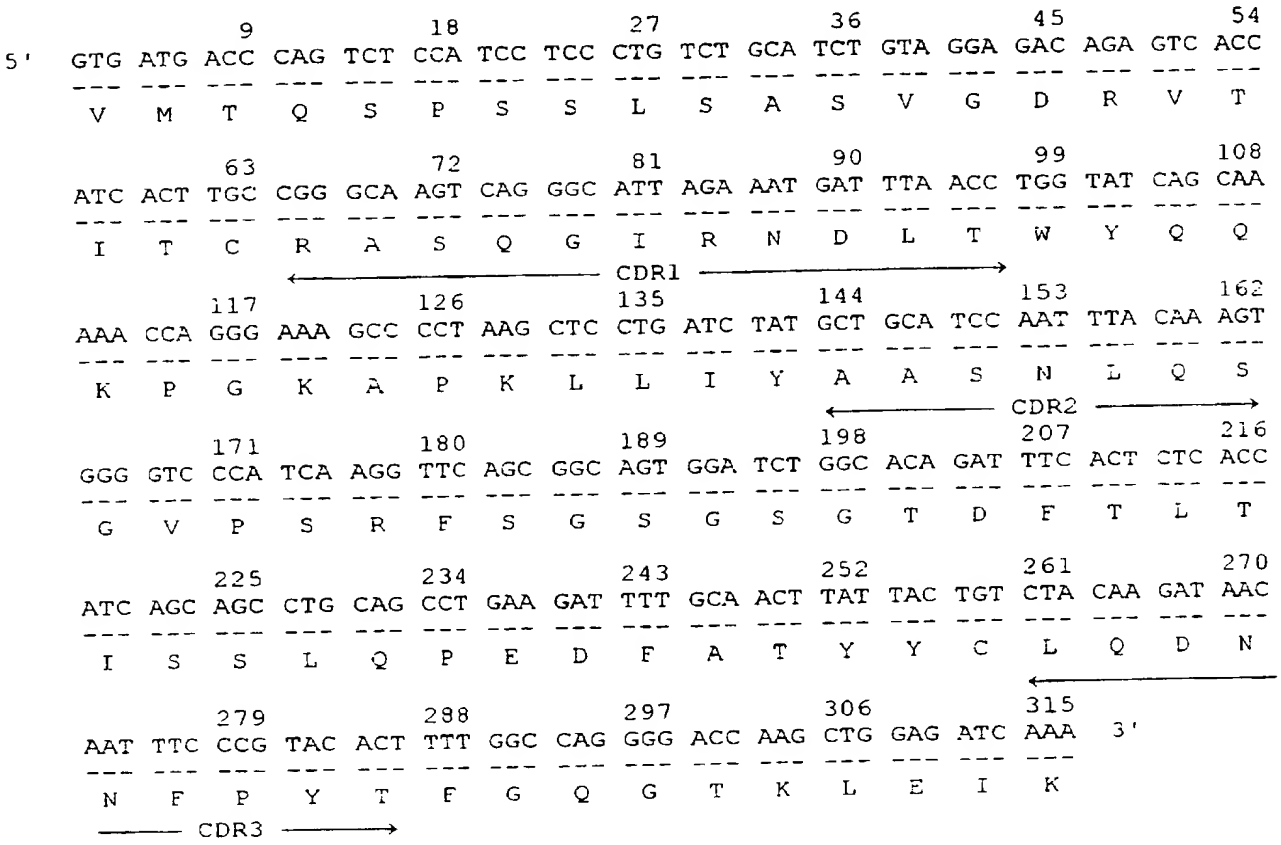


Fig. 15a

LD1/2-6-3-VH sequence

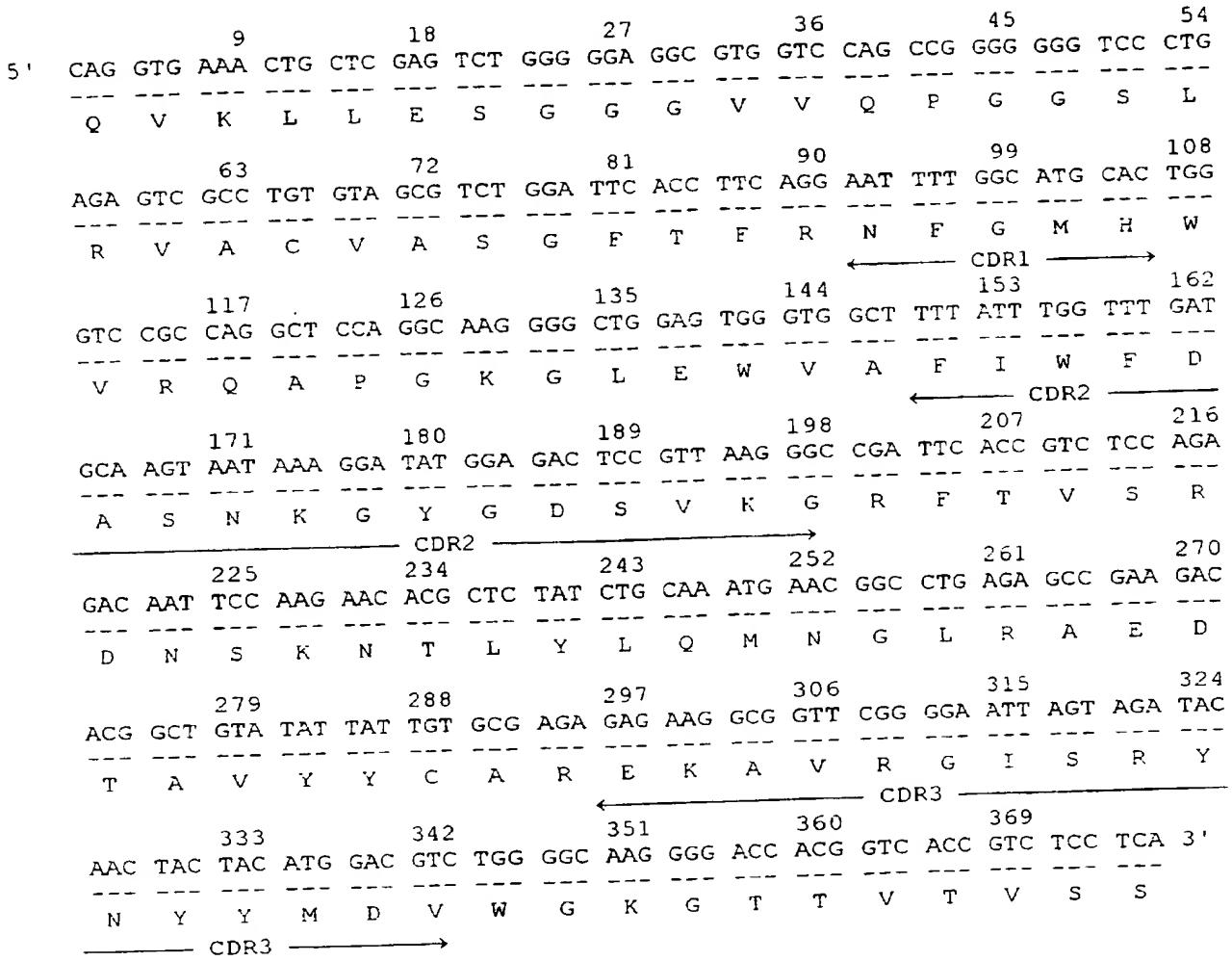


Fig. 15b

LD1/2-6-3-VL sequence

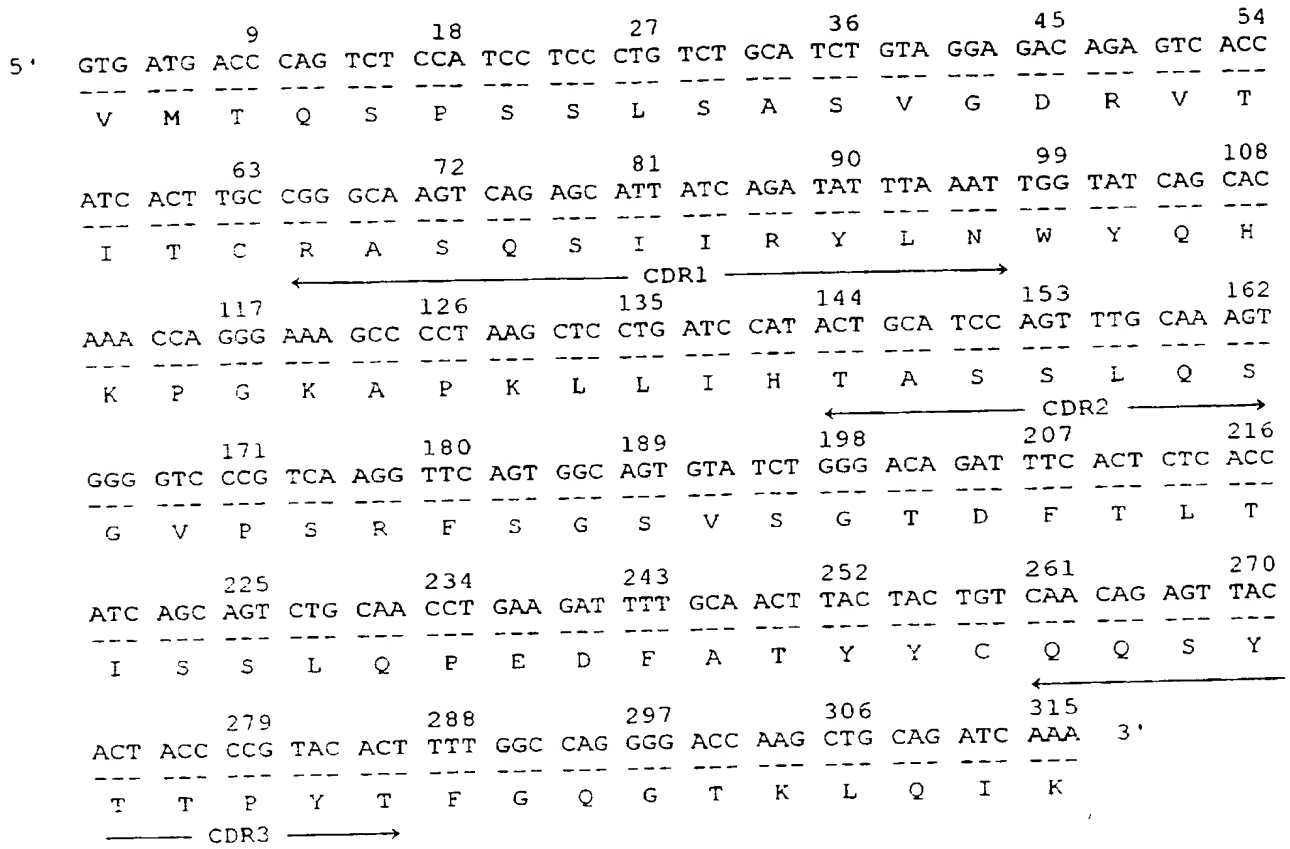


Fig. 16a

LD1/2-6-33-VH sequence

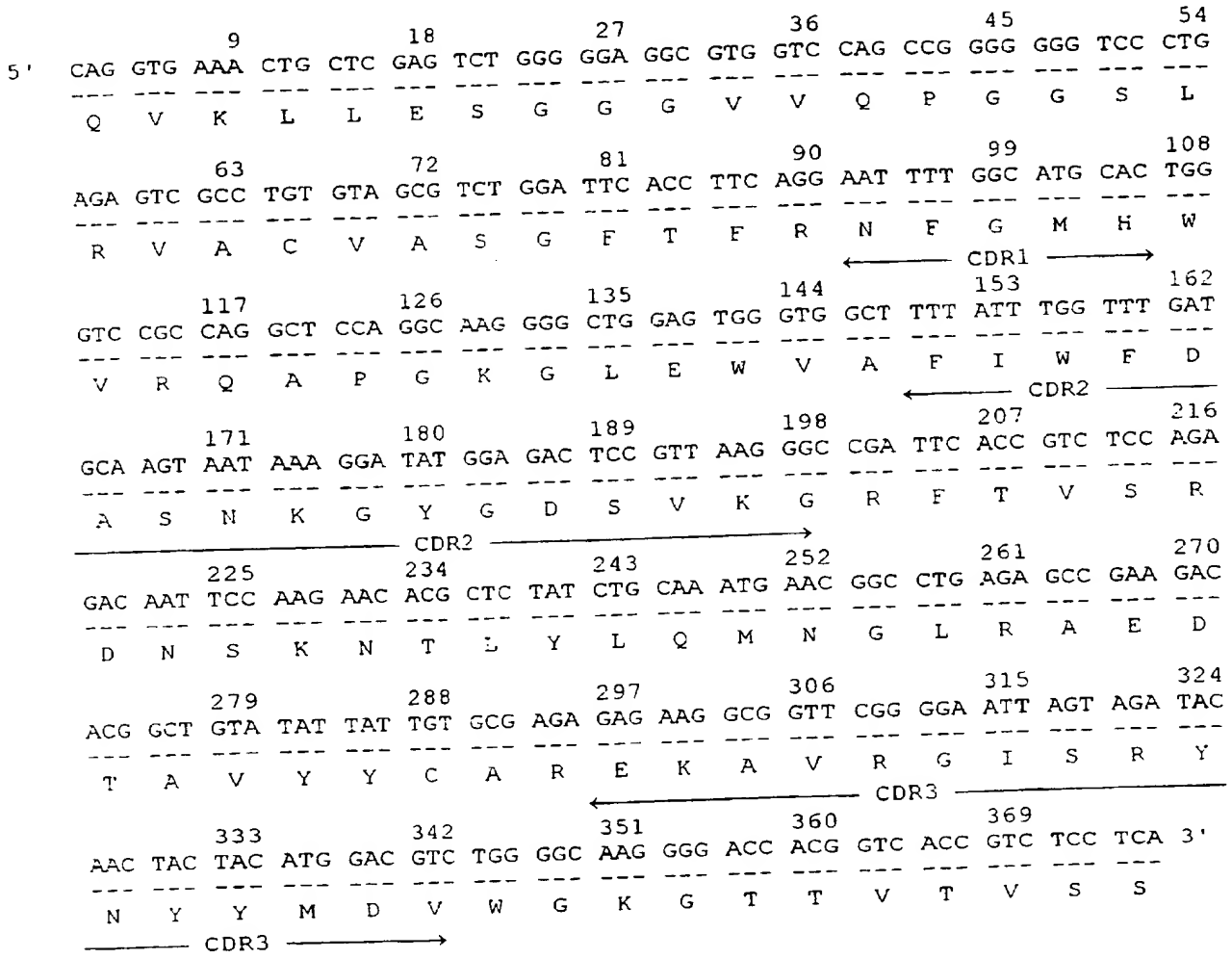


Fig. 16b

LD1/2-6-33-VL sequence

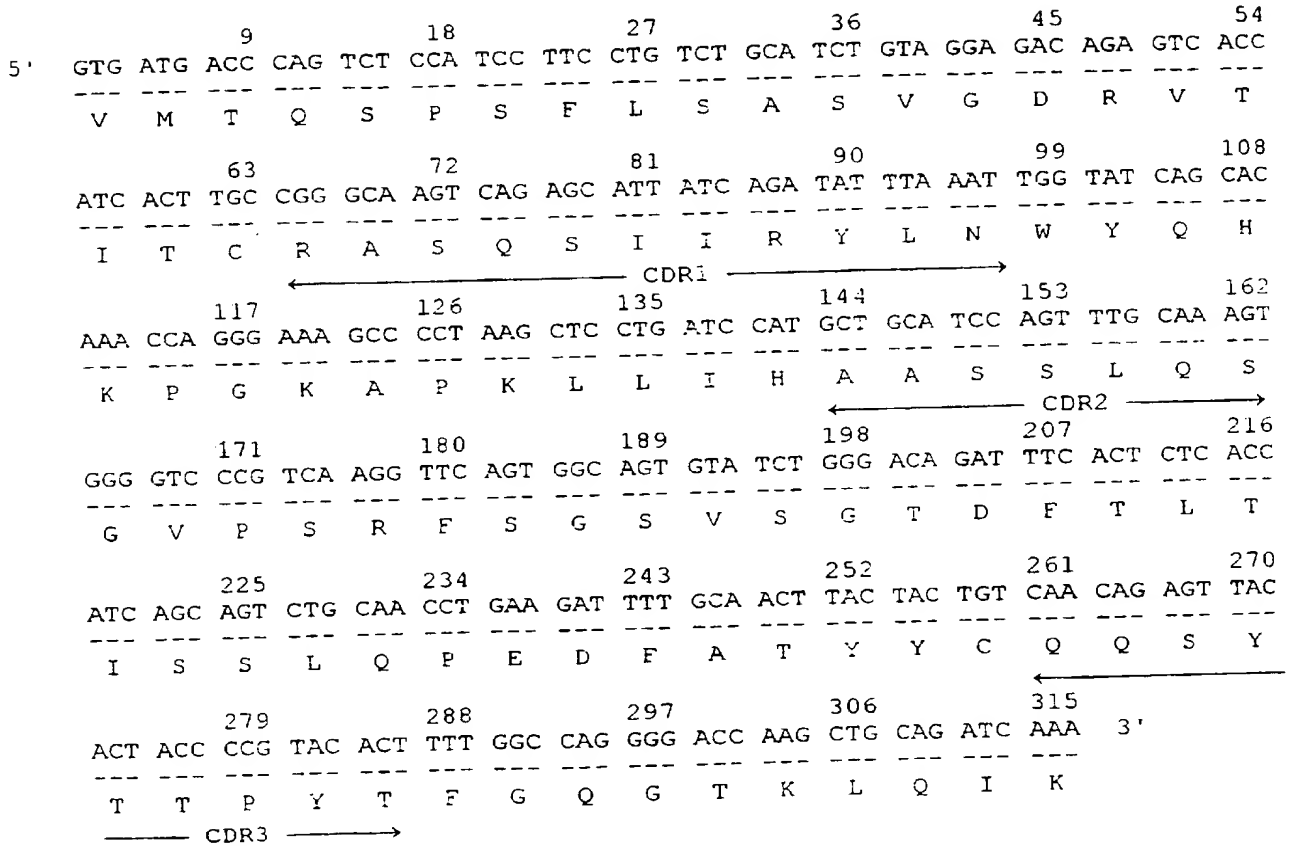
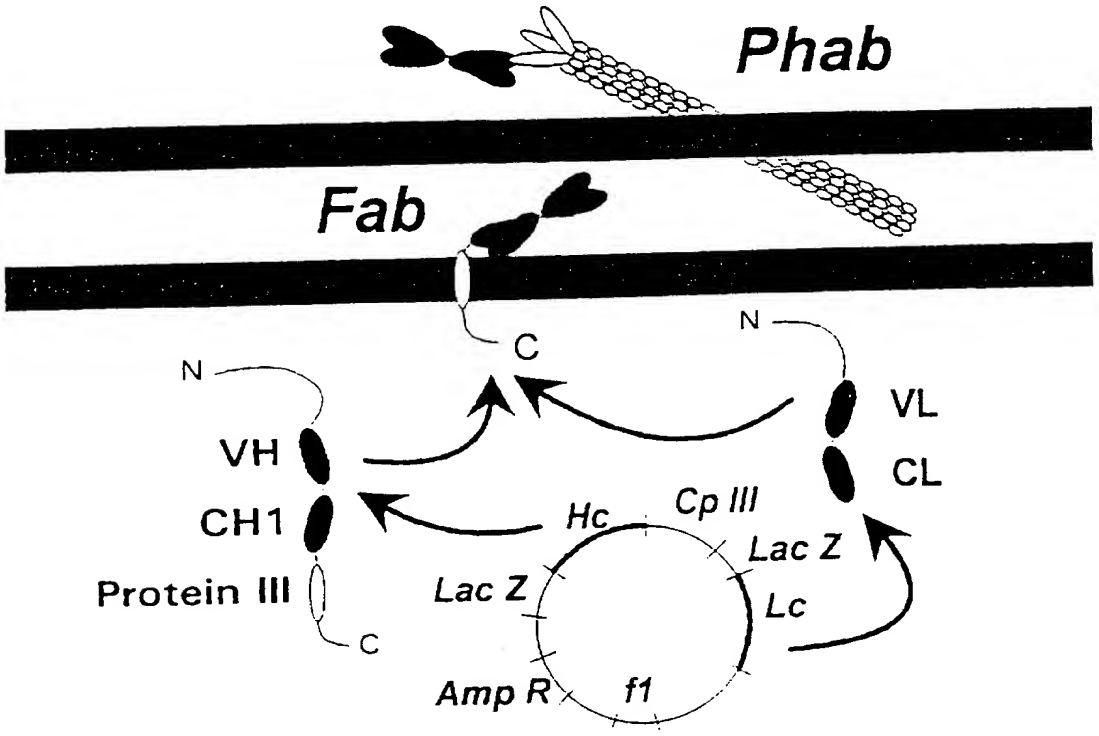


Fig. 17

The pComb3 Expression System



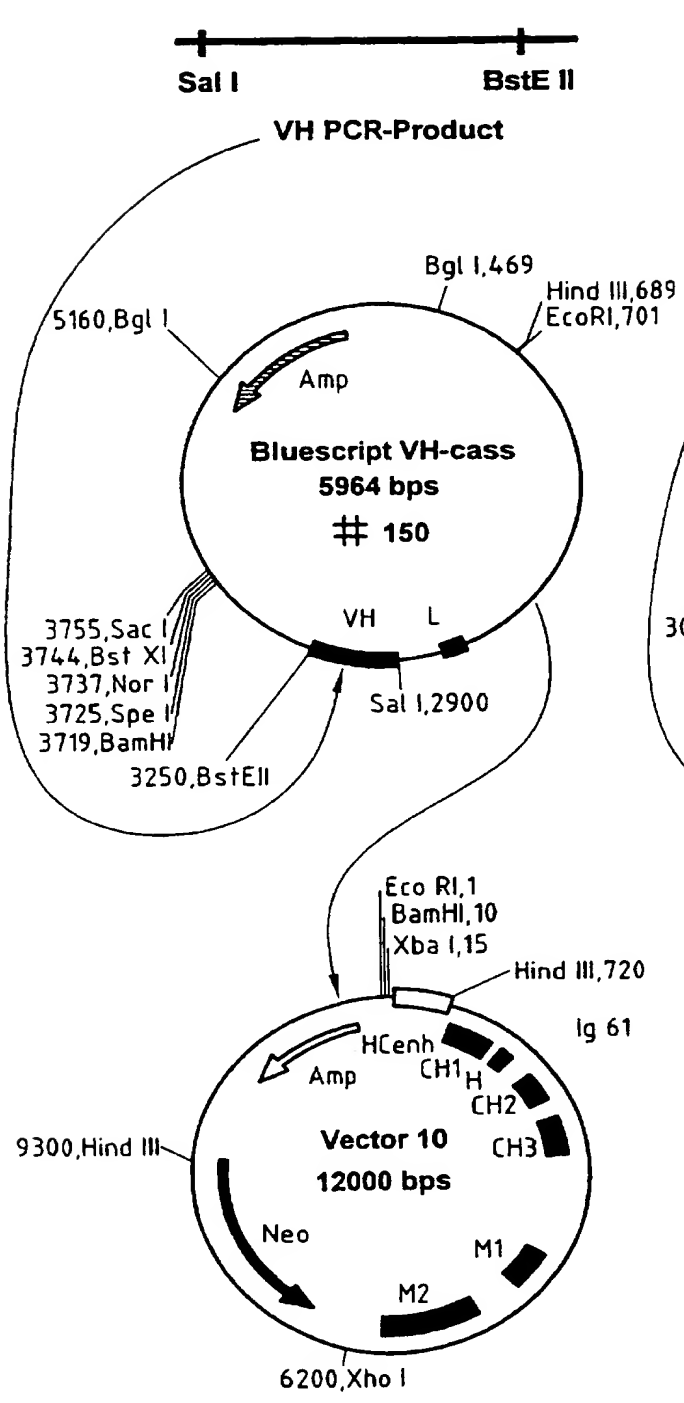


FIG. 18

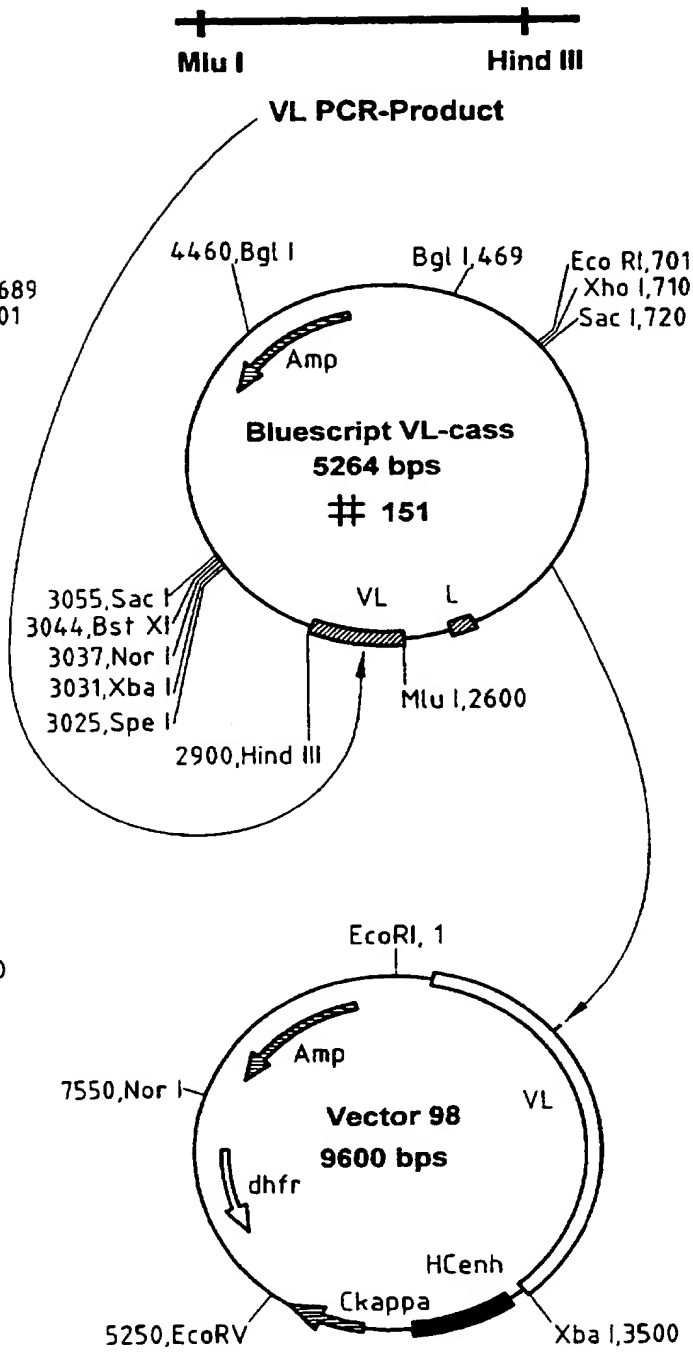


FIG. 19

INTERNATIONAL SEARCH REPORT

In. .ational Application No
PCT/EP 97/03253

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C12N15/62 C07K16/34 A61K39/395 G01N33/80

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIEGEL D. L. & SILBERSTEIN L. E.: "Expression and characterization of recombinant anti-Rh(D) antibodies on filamentous phage: a modelsystem for isolating human red blood cell antibodies by repertoire cloning" BLOOD, vol. 83, no. 8, 15 April 1994, pages 2334-2344, XP000609017 cited in the application see the whole document --- -/--	11-13, 18-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1997	14. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 97/03253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARKS J.D. ET AL.,: "Human antibody fragments specific for human blood group antigen from phage display library" BIOTECHNOLOGY, vol. 11, October 1993, pages 1145-1149, XP002018824	11
Y	see the whole document	12,13
X	DZIEGIEL M. ET AL.,: "Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D" J. IMMUNOL. METH., vol. 182, 1995, pages 7-19, XP002018825 see the whole document	11-13
X	SIEGEL D.L.: "Isolation of human anti-red blood cell antibodies by repertoire cloning" ANNALS N.Y. ACAD. OF SCIENCES, no. 764, 1995, pages 547-558, XP000609060 see the whole document	11-13,18
X	DZIEGIEL D.M. ET AL.,: "Recombinant antibody against the erythrocyte antigen, Rhesus D" J.CELL. BIOCHEM., SUPPL., 1994, page 212 XP000609398 see the whole document	11
Y	WO 91 07492 A (CENTRAL BLOOD LAB AUTHORITY) 30 May 1991 see pages 1 and 2	12,13

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/EP 97/03253

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WO 9107492 A	30-05-91	AT 148168 T	15-02-97
		AU 647054 B	17-03-94
		AU 6721490 A	13-06-91
		CA 2068222 A	14-05-91
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